PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To: MARINA T. LARSON
OPPEDAHL & LARSON LLP
P. O. BOX 5068
256 DILLION RIDGE ROAD
DILLON, CO 80435
UNITED STATES OF AMERICA

PCT

NOTIFICATION OF TRANSMITTAL OF INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of Mailing (*day/month/year*) 20 FEB 2001

IMPORTANT NOTIFICATION

Applicant's or agent's file reference MSK.P-041-WO		
International application No. PCT/US00/04445	International filing date (*day/month/year*) 03 FEBRUARY 2000	Priority Date (*day/month/year*) 04 FEBRUARY 1999 04 Aug 01 / 30 mos.
Applicant SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH		

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices)(Article 39(1))(see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer FRANK LU Dorthea Lawrence for Telephone No. (703) 308-1235

Form PCT/IPEA/416 (July 1992)★

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference MSK.P-041-WO	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US00/04445	International filing date *(day/month/year)* 03 FEBRUARY 2000	Priority date *(day/month/year)* 04 FEBRUARY 1999
International Patent Classification (IPC) or national classification and IPC IPC(7): C12Q 1/68; C12P 19/34; C12N 9/00 and US Cl.: 435/6, 91.1, 91.32, 183		
Applicant SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets.

   [√] This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 1 sheets.

3. This report contains indications relating to the following items:

   - I [X] Basis of the report
   - II [ ] Priority
   - III [ ] Non-establishment of report with regard to novelty, inventive step or industrial applicability
   - IV [ ] Lack of unity of invention
   - V [X] Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI [ ] Certain documents cited
   - VII [ ] Certain defects in the international application
   - VIII [ ] Certain observations on the international application

Date of submission of the demand 09 AUGUST 2000	Date of completion of this report 17 JANUARY 2001
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Dorothea Lawrence For FRANK LU Telephone No. (703) 308-1235

Form PCT/IPEA/409 (cover sheet) (July 1998) ★

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US00/04445

I. Basis of the report

1. With regard to the **elements** of the international application:*

[ ] the international application as originally filed

[X] the description:
pages (See Attached) , as originally filed
pages ______ , filed with the demand
pages ______ , filed with the letter of ______

[X] the claims:
pages (See Attached) , as originally filed
pages ______ , as amended (together with any statement) under Article 19
pages ______ , filed with the demand
pages ______ , filed with the letter of ______

[X] the drawings:
pages (See Attached) , as originally filed
pages ______ , filed with the demand
pages ______ , filed with the letter of ______

[X] the sequence listing part of the description:
pages (See Attached) , as originally filed
pages ______ , filed with the demand
pages ______ , filed with the letter of ______

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language ______ which is:

[ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

[ ] the language of publication of the international application (under Rule 48.3(b)).

[ ] the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/ or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

[X] contained in the international application in printed form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readable form.

[ ] The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] The statement that the information recorded in computer readable form is identical to the writen sequence listing has been furnished.

4. [X] The amendments have resulted in the cancellation of:

[X] the description, pages NONE

[X] the claims, Nos. NONE

[X] the drawings, sheets/~~fig~~ NONE

5. [ ] This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

* *Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

***Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. statement

Novelty (N)	Claims	5-15	YES
	Claims	1-4	NO
Inventive Step (IS)	Claims	6-15	YES
	Claims	1-5	NO
Industrial Applicability (IA)	Claims	1-15	YES
	Claims	NONE	NO

2. citations and explanations (Rule 70.7)

Claims 1-4 lack novelty under PCT Article 33(2) as being anticipated by Janniere et al., (Mol. Microbiology 23, 525-535, 1997).

Janniere et al., teach replication terminus for DNA polymerase I during initiation of pAMbeta1 replication. Replication of plasmid pAM beta 1 is initiated by DNA polymerase I (Pol I) and completed by DNA polymerase III holoenzyme contained in the replisome machinery. In this study they reported that initiation of DNA replication generates D-loop structures containing the nascent leading strand paired to its template (page 525, abstract) in a double stranded form and the displaced strand is in the single-stranded form (page 526, right column, third paragraph). The oligonucleotides used to characterize the segments extruded from D-loop replication intermediates have a length of from 20 to 50 bases (page 533, left column, second paragraph). The reaction involving Pol III HE was performed in the presence of ATP and four deoxynucleotides (page 533, right column). This prior art meets the limitations of the claims 1-4.

Response to Arguments

In page 2, third and fourth paragraphs of applicant's Response to Written Opinion, applicant argued that: (1)" Janniere does not disclose a replication system using proteins which are added by man to a developing D-loop. Indeed, Janniere disclose no use for the purified proteins. Furthermore, no real world application of the observation of the replication intermediates is suggested", and (2)"Janniere does not disclose the use of oligonucleotide primer or any other means to introduce a D-loop at a selected location. Indeed, in the Janniere paper, the D loop is generated as a inherent result of the addition of the polymerase, and not as a separate step prior to the assembly of the replisome. There is no targeting of the D loop to a specific initiation site adjacent to a selected target region".

The arguments have been fully considered but they are not persuasive toward the withdrawal of the rejection. First, Janniere et al., (see page 526, right column, third paragraph) (Continued on Supplemental Sheet.)

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Sheet 10

Continuation of: Boxes I - VIII

I. BASIS OF REPORT:

This report has been drawn on the basis of the description,
page(s) 1-15, as originally filed.
page(s) NONE, filed with the demand.
and additional amendments:
NONE

This report has been drawn on the basis of the claims,
page(s) 17, as originally filed.
page(s) NONE, as amended under Article 19.
page(s) NONE, filed with the demand.
and additional amendments:
Claim Page 16, filed with the letter of 13 December 2000.

This report has been drawn on the basis of the drawings,
page(s) 1, as originally filed.
page(s) NONE, filed with the demand.
and additional amendments:
NONE

This report has been drawn on the basis of the sequence listing part of the description:
page(s) 1 and 2, as originally filed.
pages(s) NONE, filed with the demand.
and additional amendments:
NONE

V. 2. REASONED STATEMENTS - CITATIONS AND EXPLANATIONS (Continued):
showed that D loop structure was generated by DNA polymerase I in the initiation of pAMbeta1 replication. it is well known that the replisome is completed by polymerase III and is required for DNA replication(Devlin, Textbook of Biochemistry with clinical correlations, third Edition, see page 671, first paragraph). Therefore, the replisome formation in the presence of assembly proteins is a inherent property of the reference of Janniere et al., and will be considered as a separate step after D loop formation. Second, Janniere et al., clearly showed the use of oligonucleotide primer to introduce a D-loop (see page 533, left column, last paragraph). Third, in response to applicant's argument that the reference failed to show certain features of applicant's invention such as "no real world application of the observation of the replication intermediates is suggested" by Janniere et al., and "there is no targeting of the D loop to a specific initiation site adjacent to a selected target region" is suggested by Janniere et al., it is noted that the features upon which applicant relies above are not recited in the claim(s). Although the claims are interpreted in light of the specification, limitations from the specification are not read into the claims.

Claims 1-5 lack an inventive step under PCT Article 33(3) as being obvious over Janniere et al., (Mol. Microbiology 23, 525-535, 1997)in view of Karet et al., (Anal. Biochem. 220, 384-390, 1994).

The teachings of Janniere et al., have been summarized previously, *supra*. This prior art meets the limitations of claims 1-4.

Janniere et al., do not disclose fluorescence labeled primer.

Karet et al., teach fluorescence labeled primer (page 384, abstract).

It would have been obvious to one having ordinary skill in the art at the time the invention was made to have used performed the method for replication of a target region of a target DNA molecule as suggested by Janniere et al., using a fluorescence labeled primer. The prior art provided by Karet et al., would have motivated one having ordinary skill in the art to perform the method for replication of a target region of a target DNA molecule using a fluorescence-labeled primer. One having ordinary skill in the art at the time the invention was made would have been a reasonable expectation of success to combine these prior arts together because all of prior art are known and are easy to use.

Claims 6-15 meet the criteria set out in PCT Article 33(2)-(4), because the prior art does not teach or fairly suggest the limitations of claims 6-15.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US00/04445

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Sheet 11

Continuation of: Boxes I - VIII

Response to Arguments

In page 2, last paragraphs of applicant's Response to Written Opinion, applicant argued that the teachings of Janniere et al., was "insufficient to cure the deficiencies of the Janniere paper".

The argument has been fully considered but it is not persuasive toward the withdrawal of the objection because, as shown above, the teachings of Janniere et al., clearly meets the limitations of the claims 1-4. There is no deficiency of the Janniere paper as suggested by applicant.

-------------------- NEW CITATIONS --------------------

DEVLIN,Thomas. Textbook of biochemistry with clinical correlation. Wiley-Liss, Inc., 605 Third Avenue, New York, NY10158-0012. 1992, pages 669-671, especially page 671.

HOME COPY

PCT REQUEST

Original (for **SUBMISSION**) - printed on 03.02.2000 02:56:14 PM

0 0-1	**For receiving Office use only** International Application No.	PCT/US 00/04445
0-2	International Filing Date	03 FEB 2000 (03.02.00)
0-3	Name of receiving Office and "PCT International Application"	PCT INTERNATIONAL APPLICATION RO/US
0-4 0-4-1	**Form - PCT/RO/101 PCT Request** Prepared using	PCT-EASY Version 2.90 (updated 15.12.1999)
0-5	**Petition** The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty	
0-6	**Receiving Office (specified by the applicant)**	United States Patent and Trademark Office (USPTO) (RO/US)
0-7	**Applicant's or agent's file reference**	MSK.P-041-WO
I	**Title of invention**	PROCESS FOR DNA REPLICATION
II	**Applicant**	
II-1	This person is:	applicant only
II-2	Applicant for	all designated States except US
II-4	Name	SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH
II-5	Address:	1275 York Avenue New York, NY 10021 United States of America
II-6	State of nationality	US
II-7	State of residence	US
II-8	Telephone No.	212-639-6181
III-1	**Applicant and/or inventor**	
III-1-1	This person is:	applicant and inventor
III-1-2	Applicant for	US only
III-1-4	Name (LAST, First)	MARIANS, Kenneth
III-1-5	Address:	c/o Office of Industrial Affairs Memorial Sloan Kettering Cancer Center 1275 York Avenue New York, NY 10021 United States of America
III-1-6	State of nationality	US
III-1-7	State of residence	US

III-2	**Applicant and/or inventor**	
III-2-1	This person is:	applicant and inventor
III-2-2	Applicant for	US only
III-2-4	Name (LAST, First)	JOING, Liu
III-2-5	Address:	c/o Office of Industrial Affairs Memorial Sloan Kettering Cancer Center 1275 York Avenue New York, NY 10021 United States of America
III-2-6	State of nationality	CN
III-2-7	State of residence	US
IV-1	**Agent or common representative; or address for correspondence** The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as:	agent
IV-1-1	Name (LAST, First)	PARSONS, Nancy
IV-1-2	Address:	Oppedahl & Larson LLP P.O. Bo 5270 611 Main Street Frisco, CO 80443-5270 United States of America
IV-1-3	Telephone No.	970-668-2050
IV-1-4	Facsimile No.	970-668-2082
IV-2	**Additional agent(s)**	additional agent(s) with same address as first named agent
IV-2-1	Name(s)	LARSON, Marina T.; OPPEDAHL, Carl
V	**Designation of States**	
V-1	Regional Patent (other kinds of protection or treatment, if any, are specified between parentheses after the designation(s) concerned)	EP: AT BE CH&LI CY DE DK ES FI FR GB GR IE IT LU MC NL PT SE and any other State which is a Contracting State of the European Patent Convention and of the PCT
V-2	National Patent (other kinds of protection or treatment, if any, are specified between parentheses after the designation(s) concerned)	CA JP US

PCT REQUEST

MSK.P-041-WO

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V-5	**Precautionary Designation Statement** In addition to the designations made under items V-1, V-2 and V-3, the applicant also makes under Rule 4.9(b) all designations which would be permitted under the PCT except any designation(s) of the State(s) indicated under item V-6 below. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit.		
V-6	**Exclusion(s) from precautionary designations**	NONE	
VI-1	**Priority claim of earlier national application**		
VI-1-1	Filing date	04 February 1999 (04.02.1999)	
VI-1-2	Number	60/118,703	
VI-1-3	Country	US	
VI-2	**Priority document request** The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) identified above as item(s):	VI-1	
VII-1	**International Searching Authority Chosen**	United States Patent and Trademark Office (USPTO) (ISA/US)	
VIII	**Check list**	number of sheets	electronic file(s) attached
VIII-1	Request	4	-
VIII-2	Description (excluding sequence listing part)	15	-
VIII-3	Claims	2	-
VIII-4	Abstract	1	abstract.txt
VIII-5	Drawings	1	-
VIII-6	Sequence listing part of description	2	-
VIII-7	TOTAL	25	
	Accompanying items	paper document(s) attached	electronic file(s) attached
VIII-8	Fee calculation sheet	✓	-
VIII-15	Nucleotide and/or amino acid sequence listing in computer readable form		separate diskette
VIII-16	PCT-EASY diskette	-	diskette
VIII-18	**Figure of the drawings which should accompany the abstract**	1	
VIII-19	**Language of filing of the international application**	English	
IX-1	**Signature of applicant or agent**	Nancy Parsons	
IX-1-1	Name (LAST, First)	PARSONS, Nancy	

PCT REQUEST

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03.02.00

10-1	**Date of actual receipt of the purported international application**	410 Rec'd PCT/PTO 03 FEB 2000
10-2	**Drawings:**	
10-2-1	Received	
10-2-2	Not received	
10-3	**Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application**	
10-4	**Date of timely receipt of the required corrections under PCT Article 11(2)**	
10-5	**International Searching Authority**	ISA/US
10-6	**Transmittal of search copy delayed until search fee is paid**	

FOR INTERNATIONAL BUREAU USE ONLY

11-1	**Date of receipt of the record copy by the International Bureau**	

PCT (ANNEX - FEE CALCULATION SHEET)

Original (for **SUBMISSION**) - printed on 03.02.2000 02:56:14 PM

(This sheet is not part of and does not count as a sheet of the international application)

0 0-1	**For receiving Office use only** International Application No.	PCT/US 00/04445		
0-2	Date stamp of the receiving Office	03 FEB 2000		

0-4 0-4-1	**Form - PCT/RO/101 (Annex)** **PCT Fee Calculation Sheet** Prepared using	PCT-EASY Version 2.90 (updated 15.12.1999)		
0-9	**Applicant's or agent's file reference**	MSK.P-041-WO		
2	**Applicant**	SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH, et al.		
12	**Calculation of prescribed fees**	fee amount/multiplier	total amounts (USD)	
12-1	Transmittal fee T	⇨	240	240
12-2	Search fee S	⇨	700	700
12-3	International fee Basic fee (first 30 sheets) b1	427		
12-4	Remaining sheets	0		
12-5	Additional amount (X)	10		
12-6	Total additional amount b2	0		
12-7	b1 + b2 = B	427		
12-8	Designation fees Number of designations contained in international application	4		
12-9	Number of designation fees payable (maximum 8)	4		
12-10	Amount of designation fee (X)	92		
12-11	Total designation fees D	368		
12-12	PCT-EASY fee reduction R	-132		
12-13	Total International fee (B+D-R) I	⇨	663	663
12-14	Fee for priority document Number of priority documents requested	1		
12-15	Fee per document (X)	15		
12-16	Total priority document fee P	⇨	15	15
12-17	**TOTAL FEES PAYABLE (T+S+I+P)**	⇨	1,618	1,618
12-19	**Mode of payment**	cheque		
12-20	**Deposit account instructions** The receiving Office:	United States Patent and Trademark Office (USPTO) (RO/US)		
12-20-2	is hereby authorized to charge any deficiency or credit any over-payment in the total fees indicated above to my deposit account	✓		

PCT (ANNEX - FEE CALCULATION SHEET)

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12-20-3	is hereby authorized to charge the fee for preparation and transmittal of the priority document to the International Bureau of WIPO to my deposit account	✓
12-21	Deposit account No.	15-0610
12-22	Date	03 February 2000 (03.02.2000)
12-23	Name and signature	PARSONS, Nancy Nancy Parsons

VALIDATION LOG AND REMARKS

13-2-2	Validation messages States	Green? More designations could be made. The following States have not been designated: AP:(GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW); EA:(AM, AZ, BY, KG, KZ, MD, RU, TJ, TM); OA:(BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG); AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CH, LI, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW. Please verify.
13-2-3	Validation messages Names	Green? Applicant 1.:Facsimile No. missing
		Green? Agent 2.: Where several first/given names are indicated, they should preferably be separated by a comma. Please verify.
13-2-6	Validation messages Contents	Yellow! The power of attorney or a copy of the general power of attorney will need to be furnished unless all applicants sign the request form.

09/ 890829

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WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12Q 1/68, C12P 19/34, C12N 9/00	A1	(11) International Publication Number: WO 00/46408 (43) International Publication Date: 10 August 2000 (10.08.00)

(21) International Application Number: PCT/US00/04445

(22) International Filing Date: 3 February 2000 (03.02.00)

(30) Priority Data:
60/118,703 4 February 1999 (04.02.99) US

(71) Applicant (for all designated States except US): SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH [US/US]; 1275 York Avenue, New York, NY 10021 (US).

(72) Inventors; and
(75) Inventors/Applicants (for US only): MARIANS, Kenneth [US/US]; Office of Industrial Affairs, Memorial Sloan Kettering Cancer Center, 1275 York Avenue, New York, NY 10021 (US). JOING, Liu [CN/US]; Office of Industrial Affairs, Memorial Sloan Kettering Cancer Center, 1275 York Avenue, New York, NY 10021 (US).

(74) Agents: PARSONS, Nancy et al.; Oppedahl & Larson LLP, P.O. Box 5270, 611 Main Street, Frisco, CO 80443-5270 (US).

(81) Designated States: CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: PROCESS FOR DNA REPLICATION

(57) Abstract

A method is provided for replicating DNA, and in particular for replicating large segments of DNA. A primer is combined with a target DNA molecule to be replicated. The primer is designed to be at least partially homologous to a known site on the target DNA, and to create a D-loop when hybridized with that site. A replisome is then assembled at the D-loop, and this replisome creates a copy of the DNA, starting at the primer binding site. By utilizing two species of D-loop primers which bind to remote sites on the DNA flanking a region to be replicated, large sections of DNA can be replicated in a manner comparable to PCR. The replicated DNA can be analyzed to detect variations in the genetic sequence of the target, for linkage mapping and as a source of longer DNA molecules having a desired sequence.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
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DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/04445

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) :C12Q 1/68; C12P 19/34; C12N 9/00
US CL :435/6, 91.1, 91.32, 183
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.1, 91.32, 183, 7.32; 436/94; 536/23.1, 23.7, 23.72, 24.3, 24.33, 25.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN and WEST
D-loop, DNA replication, pri A, replisome, helicase, primosome, primosomal proteins

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JANNIERE et al. Replication terminus for DNA polymerase I during initiation of pAMbeta1 replication: role of the plasmid-encoded resolution system. Molecular Microbiology. 1997, Vol. 23, No.3, 525-535, page 525-535, especially pages 525-527 and 533.	1-4 and 14
Y	MCGLYNN et al. The DNA replication protein PriA and the recombination protein RecG bind D-loops. J. Mol. Biol. 1997, Vol. 270, Pages 212-221, especially pages 212-214 and 217-220.	1-4, 6, 7, 10 and 14

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search	Date of mailing of the international search report
06 APRIL 2000	26 APR 2000

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KARET et al. Quantification of mRNA in human tissue using fluorescent nested reverse-transcriptase polymerase chain reaction. Anal. Biochem. 1994, Vol. 220, page 384-390, especially pages 385 and 386.	1-10 and 12-14
Y	MASAI et al., Escherichia coli PriA protein is essential for inducible and constitutive stable DNA replication. EMBO J. 1994, Vol.13, No. 22, Page 5338-5345, especially pages 5338, 5339, 5344 and 5345.	1-4, 6, 7, 10 and 14
Y	AL-DEIB et al. Modulation of recombination and DNA repair by the recG and PriA helicases of Escherchia cili K-12. J. Bacteriol. December 1996, Vol. 178, No. 23, page 6782-6789, see entire document.	1-4, 6, 7, 10, 11, 14 and 15

PROCESS FOR DNA REPLICATION

This application was supported by NIH Grant No. GM34557. The United States may have rights under this application.

This application claims priority from US Provisional Application No. 60/118,703, which application is incorporated herein by reference for those countries where such incorporation is allowed.

Background of the Invention

This application relates to a process for DNA replication, and to the application of this process for a variety of purposes.

Replication of DNA and other nucleic acids is a complex natural phenomenon which occurs within all biological systems. To facilitate the exploitation of the resources represented in the diverse genetic materials of the world's organisms, however, it is desirable to be able to replicate selected DNA sequences under more controlled conditions, for example to produce increased amounts of one sequence. Such replication of selected DNA sequences is required for a great many applications of potential scientific and industrial significance, and has been accomplished by a variety of techniques. These include cloning of the DNA sequences into plasmids or genes, and replication of the plasmid using the DNA replication mechanisms of a host organism, and amplification techniques such as PCR or ligase amplification. Cloning is capable of replicating complete gene sequences, but requires the introduction of the sequences into a host organism, and the subsequent recovery of the duplicated DNA. PCR and similar amplification techniques offer increased flexibility, including the ability to introduce labels and/or sequence variations into the replicated DNA, and avoid the use of a host organism, but are limited in the length of the sequence which can be replicated. Thus, there remains a need for a methodology which will permit the replication of long DNA molecules, while providing the flexibility associated with PCR amplification. It is an object of the present invention to provide such a methodology.

Summary of the Invention

The present invention provides a method for replicating DNA, and in particular for replicating large segments of DNA. In accordance with the invention, a primer is combined with a target DNA molecule to be replicated. The primer is designed to be at least partially homologous to a known site on the target DNA, and to create a D-loop when hybridized with that site. A replisome is then assembled at the D-loop, and this replisome creates a copy of the DNA, starting at the primer binding site. By utilizing two species of D-loop primers which bind to remote sites on the DNA flanking a region to be replicated, large sections of DNA can be replicated in a manner comparable to PCR.

The replicated DNA can be analyzed to detect variations in the genetic sequence of the target, for linkage mapping and as a source of longer DNA molecules having a desired sequence.

Brief Description of the Drawing

Fig. 1 shows the scheme used for making a double-stranded circular template DNA molecule containing a D-loop, which was used to validate the concept of the invention.

Detailed Description of the Invention

The present invention provides a method for the controlled replication, generally *in vitro*, of selected regions of DNA. In accordance with the invention, replication of a target region of a target DNA molecule is accomplished by:

(a) introducing a D-loop into the target DNA molecule at a selected initiation point adjacent to the target region;

(b) assembling a replisome at the D-loop; and

(c) providing DNA monomers (dNTPs) and ATP, whereby the target region is replicated. ATP is preferably provided at concentrations in excess of about 1 mM. ATP is required because the formation of a processive DNA polymerase complex requires ATP hydrolysis and also because DnaB, the DNA helicase, requires concentration in excess of 1 mM to be maximally active.

Introduction of a D-loop at a selected initiation site in duplex DNA can be accomplished using an oligonucleotide primer which hybridizes with double-stranded DNA at a selected initiation site. The non-hybridized strand is displaced to create the D-loop. D-loop formation can be driven by the homologous pairing enzyme, RecA, as has been described in the literature. See, McEntee et al., *Proc. Nat'l Acad. Sci. (USA)* 76: 2615-2619 (1979), which is incorporated herein by reference. D-loop formation could also be driven by other methods, for example heating at a moderately high temperature (for example 75-80°C) may be enough to drive annealing, particularly in regions rich in A+T bases.

The oligonucleotide primer which is used for generation of the D-loop generally has a length of from 20 to about 50 bases. The primer is selected to be substantially complementary to one of the two strands of the target DNA duplex at the initiation site. As used herein, the term "substantially complementary" refers to a primer which will hybridize with the target DNA duplex under conditions of moderately high stringency. However, it will be appreciated that RecA mediated hybridization, if employed, is an enzymatic strand-pairing reaction, and that conditions normally used for DNA-DNA hybridization (e.g. 0.6 M NaCl) would actually be inhibitory. Thus the precise conditions corresponding to "moderately high stringency" may vary depending on the methodology used to drive the annealing. In a general sense, however, the term "substantially complementary" includes (1) primers which are perfectly complementary to the target DNA molecule, (2) primers which are complementary for most of their length, but which include one or several mismatches from perfect complementarity, although not enough mismatches to significantly reduce hybridization specificity; and (3) degenerate primers which include several bases at a given site to accommodate a multiplicity of common alleles in the target DNA. The use of mismatched primers may result from the presence of a mutation in the initiation site, or the mismatch may be intentionally selected for introduction of a desired sequence variation into the replicated DNA.

The primers used in the invention may also include one or more non-hybridized regions for the purpose of introducing a desired additional sequence into the replicated DNA. For example, this additional sequence may be a sequence which introduces a restriction site near the end of the replicated DNA to facilitate insertion of the replicated copies into other DNA molecules. Preferred restriction sites will be those recognized by

rare-cutting restriction enzymes which generally recognize 8-base sequences, or intron-homing endonucleases such as PI-SceI from yeast which recognizes a 31-base pair sequence. This will reduce the likelihood of cleavage occurring within the replicated DNA at other than the intended cleavage site.

In an alternative embodiment of the invention useful with single-stranded templates, the primer used comprises a 3'- and a 5' region which are substantially complementary to portions of the target DNA template, and a central non-complementary region which forms a D-loop when the primer is hybridized with the target DNA. A second primer which is complementary is used to form the invading strand of the D-loop. Similar variations for insertion of cleavage sites etc, may be incorporated in the structure of such primers.

The primers used in the method of the invention may also include a detectable label or capture moiety. Suitable detectable labels and capture moieties are well known in the art as comparable materials are used in PCR, nucleic acid sequencing, and hybridization-based assays. Specific, non-limiting examples of suitable labels and/or capture moieties include fluorescent dyes such as fluorescein, Texas Red or cyanine dyes; enzyme labels such as alkaline phosphatase; and capturable labels such as biotin. Nucleic acid tails which specifically interact with a known capture sequence can also be employed.

In a preferred embodiment of the invention, the primer is combined with target double-stranded DNA under conditions suitable for hybridization and in the presence of the enzyme RecA, which results in the formation of a D-loop at the site of primer binding. Unlike common *in vitro* processes such as PCR, which utilize bacterial polymerases of inherently low processivity, the present invention utilizes replisomes. Replisomes are multi-protein associations which form at a replication fork and act in concert to replicate DNA. Replisomes provide much greater processivity than polymerases used for PCR. For example, the *E. coli* replisome can synthesize pieces of DNA at least as long as a megabase (1×10^6 nucleotides). The fidelity of copying is also quite high, with the *E. coli* replisome making fewer than 1 mistake in 10^8 nucleotides synthesized. Furthermore, unlike PCR, replisomes are substantially insensitive to regions of secondary structure in the DNA template. Thus, utilization of replisomes offers numerous advantages over the use of polymerases.

Replisomes include proteins which perform a variety of functions. Replication of DNA using replisomes depends on an initial unwinding of the DNA duplex at an origin of replication, and the continued unwinding along the strands as the replication process proceeds. This unwinding is carried out by DNA helicases. The resultant regions of single-stranded DNA are stabilized by the binding of single-stranded DNA-binding proteins which are also part of the replisome. The stabilized single-stranded regions are then accessible to the enzymatic activities of polymerases enzymes required for replication to proceed.

Replisomes have been shown to be substantially self assembling. Thus, when the necessary proteins are present under appropriate conditions, the replisome will assemble. We have found that this assembly will occur at a D-loop. A preferred combination of proteins for formation of a replisome in accordance with the present invention includes the following proteins:

PriA, PriB, PriC, DnaT, DnaB, DnaC (primosomal proteins);
single-stranded DNA-binding protein (SSB); and
DNA polymerase III holoenzyme (Pol III HE).

An alternative combination utilizes the mutant protein DnaC810, (described below) in place of PriA, PriB, PriC and DnaT.

The preparation and recovery of these various proteins is well described in the art, including the art cited below which is incorporated herein by reference. Pol III HE may be used in a form recovered directly by purification from *E. coli*, or as a combination of Pol III* and the β subunit. Pol III HE may also be reconstituted from individually overexpressed and purified subunits. These subunits are α (DnaE), ε (DnaQ), θ (HolE), β (DnaN), τ (DnaX, full length), γ (DnaX, truncated), δ (HolA), δ′ (HolB), χ (HolC) and ψ (HolD). Preparation of Pol III HE is described in US Patents Nos. 5,668,004 and 5,583,026 which are incorporated herein by reference for those countries in which such incorporation is permitted.

Replisomes have been found to initiate DNA replication at the site of a D-loop. Thus, the D-loop formed by the interaction of the primer with the target DNA molecule serves as the initiation site for the replication process in accordance with the invention. When appropriate nucleic acid monomers (i.e., deoxynucleotide triphosphates,

dATP, dCTP, dGTP and dTTP) and ATP are available, a copy of the strand of the DNA molecule to which the primer hybridizes is produced. The length of replicated material which can be produced in this way is much greater than the length which can be produced using PCR or comparable techniques, with lengths in excess of 5000-500,000 bases being readily attainable. Thus, the method provides the ability to make copies of entire large genes, including both intron and exon sequences.

As will be apparent to persons skilled in the art, a person making copies of DNA will generally be interested in obtaining those copies of a particular region of the DNA, which is referred to herein as the "target region." The target region may be a particular gene, or a particular portion of a gene depending on the use for which the copied DNA is intended. The ability to produce copies of very large numbers of bases changes the practical limits on the proximity between the primer and the target region from those which are usually observed in the PCR and comparable methods. Thus, while the initiation site must be "adjacent" to the target region, this means only that the initiation site must be close enough to and on the correct side of the target region such that a replisome assembled at the D-loop will copy the DNA of the target region.

In a preferred embodiment of the invention, two primers are utilized. The first primer is as described above, and hybridizes with a first strand of a double stranded DNA duplex. The second primer also is a substantially complementary oligonucleotide primer, but it hybridizes to the second strand of the DNA duplex at a second initiation site located on the other side of the target region. Thus, the two primers flank the target region, in the same manner that PCR primers flank a region to be amplified. Further, the same principle which leads to amplification of just the region bounded by PCR primers, leads to creation of much larger pieces of replicated DNA spanning the region between the two initiation sites using the method of the invention, although the efficiency may not be as great as achieved with PCR. This reduced efficiency is less of a problem than one might expect, however, since the large size of the replicated DNA makes them inherently more detectable than small fragments. On the other hand, since the process of the invention works on double-stranded DNA, it is not necessary to separate the strands of the target and the newly replicated DNA before proceeding with the next cycle.

While the large size of the replicated DNA offers advantages for purposes of detection, it may also pose problems. Very large DNA molecules (i.e., those that are hundred of kilobases in length) are fragile, and may be broken if manipulated in simple solutions. Thus, production of fragments of such lengths, and meaningful analysis of the lengths of such fragments may require that the reaction be performed in a supporting matrix, such as an agarose gel. Replicated DNA can be transferred out of the supporting matrix, for example for introduction into a matrix for separation based on size by electrophoresis.

DNA replicated in accordance with the invention may be utilized for a variety of purposes. First, the replicated DNA may be used as a source of genetic material to be spliced into still larger nucleic acid constructs, including plasmids, cosmids, viral vectors etc., to facilitate expression of the replicated DNA in a suitable host system. Such splicing can be facilitated by the incorporation of restriction sites near then ends of the replicated DNA as discussed above. When two primers are utilized, restriction sites can be introduced at both ends of the replicated DNA.

Second, the replication of DNA in accordance with this method can be used as part of a method for detecting genomic rearrangements in a target DNA sequence. In such a method, a D-loop is introduced into the DNA at a selected initiation point, a replisome is assembled at the D-loop, and the DNA is copied to produce sufficient numbers of copies for analysis. The copied product is analyzed to detect variations in size or organization of the copied material using size-specific separations, hybridization probes and other standard analytical techniques. It will be appreciated that the use of size-specific separations requires the production of a product of defined lengths, and thus will generally require the use of the two primer embodiment discussed above. On the other hand, where the analysis involves the measurement of the interaction of the DNA with a labeled or immobilized probe, the replication of multiple copies of a single strand of the DNA, without amplification, may be sufficient.

Third, the method can be used to facilitate linkage mapping. For example, the method can be used in the circumstance where two chromosomal markers are known to be near one another, but where the exact distance separating them is not known. D-loop oligonucleotide primers are synthesized for each marker for both the DNA strands. Combinations of the primers are used to replicate the region between the two markers, and

the size of the product formed reflects the chromosomal distance between the two markers. The method may also be used to map unlinked genes, and markers such as RFLPs, SNIPs and ESTs.

To demonstrate the ability of the replisomes to assemble at a D loop and replicate the DNA, we used a small bacteriophage DNA molecule as a model system as described in the following non-limiting examples. The conditions for replisome assembly and DNA replication can be extended to use with larger molecules, and with substantially complementary primers as discussed above.

EXAMPLE 1

Preparation of DNA Replication Proteins

To prepare DnaC810, a *dnaC810* open reading frame was constructed by splicing overlap extension polymerase chain reaction and cloned into the *Nde*I site of the pET11C overexpression plasmid (Novagen). Overexpression and purification of DnaC810 was as for the wild type protein.

PriA, PriB, PriC, DnaT, DnaB and DnaC were purified by the methods described in Marians, K.J. *Methods Enzymol.* 262: 507-521 (1995). SSB was purified using the procedures described in Minden and Marians, *J. Biol. Chem.* 260: 9316-9325 (1985). The DNA polymerase III holoenzyme was either reconstituted from Pol III* and β subunit as described by Wu et al. *J. Biol. Chem.* 267: 4030-4044 (1992) or from purified subunits as described in Marians et al., *J. Biol. Chem.* 273: 2452-2457 (1998).

EXAMPLE 2

To validate the operability of the inventive concept, a double-stranded circular template DNA was prepared in accordance with the steps shown in Fig. 1. A 100 nt-long oligonucleotide primer (Seq. ID No. 1) was annealed to f1R408 viral DNA (Russell et al., *Gene* 45: 333-339 (1986)). The central 42 nt of this oligonucleotide are non-homologous with the template, thus forming a D-loop in the resulting heteroduplex. Incubation of the heteroduplex with DNA Polymerase III holoenzyme in the presence of SSB and DNA monomers resulted in the extension of the primer and the formation of a nicked form II DNA with a 42 nt-long bubble region. During the last two minutes of this

incubation, ddTTP and ddATP were introduced at concentrations 20-fold higher than dTTP and dATP to ensure that complementary strand synthesis could not be extended further. After phenol extraction and ethanol precipitation, the DNA products were purified by electrophoresis through native agarose gels. Complete form II bubble DNA was recovered from the gel and a [5'-^{32}P] minus strand oligonucleotide (Seq. ID. No. 2) was then annealed to the D loop form II template. The template was then gel filtered through Biogel A5M to remove unannealed oligonucleotide and unincorporated [γ-^{32}P] ATP.

EXAMPLE 3

Reaction mixtures (12 μl) containing 50 mM Hepes-KOH (pH 8.0), 10 mM MgOAc, 10 mM DTT, 80 mM KCl, 200 μg/ml bovine serum albumin, 2 mM ATP, 40 μM dNTPs, 0.42 nM [^{32}P] form II D loop DNA template, 0.5 μM SSB, 225 nM DnaC, 30 nM DNA polymerase III holoenzyme, PriA, PriB, PriC, DnaT and DnaB were incubated at 37°C for 10 minutes. To test the sufficiency of various combinations of proteins to replicate the template prepared in Example 2, reactions were also performed in which one of the proteins (PriA, PriB, PriC, DnaT, DnaC and DnaB) was omitted in each reaction mixture. As controls, template alone and template with the holoenzyme alone were also evaluated. Reactions were terminated by the addition of EDTA to a concentration of 25 mM and NaOH to a concentration of 50 mM. The reaction products were evaluated by electrophoresis at 2 V/cm for 20 hours at room temperature through horizontal 0.7% alkaline agarose gels using 30 mM NaOH, 2 mM EDTA as the electrophoresis buffer. The gels were neutralized, dried and analyzed by autoradiography.

The electrophoresis gels showed that incubation of the D-loop template, the seven primosomal proteins, SSB and DNA polymerase III holoenzyme resulted in extension of the invading strand oligonucleotide (42 nt, Seq. ID. No. 2) to the full length template size (6.4 kb). The efficiency of the reaction varied, but generally 15-30% of the invading strand could be elongated to full length in a 10 minute incubation. The reaction exhibited an absolute requirement for all of the primosomal proteins except PriC. Omission of this protein resulted in a decrease in DNA synthesis to one-third that of the complete reaction. This observation was similar to those reported for replication on different templates. Ng et al., *J. Biol. Chem.* 271: 15642-15648 (1996). Some extension of the invading strand by the

holoenzyme alone could be observed, but this was suppressed by the presence of PriA. If the invading strand was omitted from the reaction, and [α-^{32}P] dATP was included, no DNA replication could be observed.

EXAMPLE 4

Because DNA helicases were being introduced to the DNA during primosome assembly, extension of the invading strand could result from one of two processes: either (1) assembly of a *bona fide* replication fork at the D loop followed by elongation of the leading strand coupled with unwinding of the duplex DNA template, or (2) uncoupled unwinding of the template DNA leaving an oligonucleotide annealed to the viral single stranded DNA that could be elongated in a primer extension reaction by the polymerase. We previously showed that coupled replication fork action requires a protein-protein interaction between DnaB and the τ subunit of the holoenzyme. Kim et al.., *Cell* 84: 643-650 (1996). In the presence of this interaction, replication forks could move rapidly, at nearly 1000 nt/sec, whereas in its absence, the polymerase becomes stuck behind a slow-moving helicase and replication fork progression proceeds at only about 30 nt/sec.

To evaluate the mechanism active in the replication of DNA in the method of the invention, the speed of elongation of the invading strand was assessed in the presence and absence of τ using holoenzyme reconstituted from individual purified subunits. Ten second time points were taken from the start of the reaction, and the elongated products were examined on denaturing gels. Full length material could be observed in the presence of τ after 10 seconds, whereas even after 60 seconds no full length material was observed in its absence. This corresponds to a rate of replication fork progression in the presence of τ of 600-700 nt/sec, similar to what has been observed in the past for other replication systems. Mok et al., *J. Biol. Chem.* 262: 16644-16654 (1987). Thus, we conclude that *bona fide* replication fork assembly occurs at the D loop on the template in the presence of primosomal proteins, SSB and the holoenzyme.

EXAMPLE 5

All of the phenotypes of *priA* null mutations can be suppressed by mutated *priA* alleles that encode PriA proteins that are no longer ATPases or DNA helicases, but still

catalyze primosome assembly. Zavitz et al., *J. Biol. Chem.* 267: 6933-6940 (1992). These mutations are substitutions in the invariant Lys in the Walker A box nucleotide-binding motif. If the PriA-dependent replication fork assembly described here were relevant to what happened in the cell, we would expect these mutant proteins to substitute fully for wild-type PriA in the replication reaction. To test this, three mutant proteins, having the K230R, K230A and K230D substitutions were tested. All three supported replication on the D loop to a greater extent than the wild-type protein. This same type of improved activity in the mutant proteins has been observed in other systems (Zavitz, *supra*), and may arise because the mutant proteins remain bound to the site of DNA binding, providing a better target than the wild-type protein that can move off the site because of its helicase activity.

EXAMPLE 6

E. coli strains carrying *priA* mutations are very difficult to grow. They are rich-media sensitive, form huge filaments, and have a viability roughly one-hundredth that of the wild-type. Sandler et al., *Genetics* 143: 5-13 (1996); Nurse et al., *J. Bacteriol.* 6686-6693 (1991); Masai et al., *EMBO J.* 13: 5338-5345 (1994). Suppressor mutations that restore viability, as well as ablate constitutive induction of the SOS response and the defects in homologous repair of UV-damaged DNA, arise overnight after transduction of the *priA2:kan* allele into fresh recipient cells. The mutations map to *dnaC*. (Sandler, *supra*). DnaC forms a complex with DnaB in solution (Wicker et al., *Proc. Natl. Acad Sci. (USA)* 72: 921-925 (1975), and is required for the efficient transfer of DnaB to DNA in the presence of other replication protein. Marians et al., *Ann. Rev. Biochem.* 61: 673-719 (1992). In order to assess the biochemical properties of these altered DnaC proteins, one such suppressor allele, *dnaC810*, was molecularly cloned into an expression plasmid and the mutant protein purified as described in Example 7, *infra*.

Strains carrying *dnaC810* no longer require PriA for viability. This suggests that if the essential role for PriA in cellular metabolism was to catalyze assembly of replication forks at recombination intermediates, DnaC810 must be able to bypass the requirement for PriA to recognize the D loop and nucleate the assembly of a primosome. Accordingly, we tested whether DnaC810 alone could direct transfer of DnaB to the D loop template DNA.

In the presence of SSB and the holoenzyme, the combination of wild-type DnaC and DnaB did not support elongation of the invading strand of the D loop. On the other hand, DnaC810 was clearly able to load DnaB to the D loop on the template in the absence of the other primosomal proteins, as evidenced by the elongation of the invading strand to full length. Thus, the E176G substitution in DnaC810 represents a true gain of function mutation that allows bypass of the DnaB loading pathway that involves PriA, PriB, PriC and DnaT and permits a reduction in the number of proteins necessary for the practice of the present invention.

Interestingly, the relative efficiencies of the replication reaction catalyzed in the presence of DnaC810 and DnaB varied compared to the reaction catalyzed by the complete set of primosomal proteins. At 80 mM KCl, the DnaC810 reaction was 5- to 10-fold more efficient. However, at 600 mM potassium glutamate, the reaction catalyzed by the complete set of proteins was more efficient by a factor of 2. While not intending to be bound by a particular mechanism, this difference may arise from differences in the relative stability of intermediate complexes that are formed during the loading of DnaB to DNA.

EXAMPLE 7

Construction of Plasmid pET11c-dnaC810—A *dnaC810* open reading frame (ORF) was made by two-step overlapping polymerase chain reaction (PCR) Morton et al., *Gene* 77: 61-68 (1989). The N-terminal coding region of *dnaC810* was PCR amplified using plasmid pET11c-*dnaC* (Marians, K.J, *Methods Enzymol.* 262:m 507-521 (1995)) as a template and two flanking primers:

(i) the NdeI primer (Seq. ID No. 3), which carries a *Nde*I site at the *dnaC* initiator codon, and

(ii) the AgeI′ primer (Seq. ID. No. 4), which carries the designed point mutation (E176G, GAA→GGT). The C-terminal coding region of *dnaC810* was also PCR amplified using plasmid pET11c-*dnaC* as a template and two different flanking primers:

(i) the AgeI primer (Seq. ID No. 5), which is complementary to the AgeI′ primer and

(ii) the BamHI primer (Seq. ID No. 6), which carries a *Bam*HI site just downstream of the *dnaC* stop codon. These overlapping N- and C-terminal fragments were gel purified after PCR and further PCR extended and amplified with the two flanking NdeI and BamHI

primers. The gel purified *dnaC810* ORF fragment was digested with *Nde*I and *Bam*HI and ligated with *Nde*I- and *Bam*HI-digested pET11c plasmid DNA to give pET11c-*dnaC810*.

Purification of DnaC810—Because of the extreme overproduction, DnaC810 was followed during purification by SDS-PAGE. BL21(DE3)pLysS carrying pET11c-*dnaC810* was grown in 12 l L Broth (Mainatis et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1982)) containing 0.4% glucose and 300 mg/ml ampicillin to OD_{600} = 0.4 and then induced in the presence of 1 mM IPTG for 3 h. Cells were chilled, pelleted by centrifugation, and resuspended in 50 mM Tris-HCl (pH 8.4 at 4 °C) and 10% sucrose. The cell suspension (50 ml) was adjusted to 150 mM KCl, 20 mM EDTA, 5 mM dithiothreitol, 0.02% lysozyme, and 0.1% Brij 58 and incubated at 0 °C for 10 min. This suspension was centrifuged at 100,000 × g for 1 h (Sorvall T865 rotor). The supernatant (fraction 1, 65 ml, 3510 mg protein) was adjusted to 0.04% polymin P by dropwise addition of a 1% solution. The precipitate was removed by centrifugation at 47,000 × g in a Sorvall SS-34 rotor for 30 min. The supernatant was further subjected to $(NH_4)_2SO_4$ fractionation (50% saturation) by the addition of solid. The resulting protein pellet was collected by centrifugation at 47,000 × g in a Sorvall SS-34 rotor for 30 min. The protein pellet was resuspended in 8 ml of buffer A [50 mM Tris-HCl (pH 7.5 at 4 °C), 1 mM EDTA, 5 mM dithiothreitol, 20% glycerol, 0.01% Brij 58] + 50 mM NaCl to give fraction 2 (13 ml, 1108 mg protein). Fraction 2 was dialyzed against 2 l of buffer A + 50 mM NaCl for 12 h and then loaded onto a 100-ml DEAE-cellulose column (4 cm x 20 cm) that had been equilibrated previously with buffer A + 50 mM NaCl. The column was washed with 200 ml of buffer A + 50 mM NaCl. Fractions (15 ml) of the flow-through and wash that contained protein were pooled to give fraction 3 (81 ml, 363 mg protein). Fraction 3 was loaded directly onto a 35-ml SP-Sepharose FF column (formed in a 60-ml disposable syringe) that had been equilibrated previously with buffer A + 50 mM NaCl. The column was washed with 200 ml of buffer A + 50 mM NaCl and protein was then eluted with a 350-ml linear gradient of 50-300 mM NaCl in buffer A. DnaC810 eluted at 175 mM NaCl (fraction 4, 24 ml, 25 mg protein). Fraction 4 was then loaded directly onto a 6-ml hydroxylapatite column (packed in a 10-ml disposable syringe) that had been equilibrated previously with buffer A + 200 mM NaCl. The column was washed with 12 ml of equilibration buffer and protein was eluted with a 60-ml linear gradient of 0-400

mM $(NH_4)_2SO_4$ in buffer A + 200 mM NaCl. DnaC810 eluted at 150 mM $(NH_4)_2SO_4$ to give fraction 5 (5.2 ml, 16.5 mg protein). Fraction 5 was concentrated bydialyzing against buffer A + 50 mM NaCl + 30% polyethylene glycol 20,000 and loaded onto a 125-ml Superdex-200 FPLC column that had been equilibrated with buffer A + 50 mM NaCl. The column was eluted at 1 ml/min. Fractions (1 ml) containing DnaC810 were pooled to give fraction 6 (7.5 ml, 9.2 mg protein). Fraction 6 was then loaded onto a 3-ml phosphocellulose column that had been equilibrated with buffer A + 50 mM NaCl. The column was washed with 6 ml of equilibration buffer and protein was eluted with a 60-ml linear gradient of 50-400 mM NaCl in buffer A. DnaC810 eluted at 250 mM NaCl (Fraction 7, 3.5 ml, 5.2 mg protein).

Sequence Listing

(Seq. ID No. 1)	ACATACATAA AGGTGGCAAC GCCATTCGAA
	ATGAGCTCCA TATGCTAGCT AGGGAGGCCC
	CCGTCACAAT CAATAGAAAA TTCATATGGT TTACCAGCGC
(Seq. ID No. 2)	ATATAAAAGA AACGCAAAGA CACCACGGAA
	TAAGTTTATT TT
(Seq. ID No. 3)	TAATGCAGGC CATATGAAAA ACGTTGGCGA CCTG
(Seq. ID No. 4)	TCGTATTTCG AACCGGTCTG CACG
(Seq. ID No. 5)	CGTGCAGACC GGTTCGAAAT ACGA
(Seq. ID No. 6)	TTAAGCACTG GGATCCTTAA TACTCTTTAC CTGTTAC

CLAIMS

1. A method for replication of a target region of a target DNA molecule comprising the steps of:

(a) introducing a D-loop into the target DNA molecule at a first initiation point adjacent to the target region;

(b) assembling a replisome at the D-loop; and

(c) providing DNA monomers and ATP to the replisome, whereby the target region is reproduced.

2. The method of claim 1, wherein the target DNA molecule is a duplex DNA.

3. The method of claim 2, wherein the step of introducing a D-loop is performed by hybridizing the duplex DNA molecule with a first oligonucleotide primer which is substantially complementary to the first initiation site.

4. The method of claim 3, wherein the first oligonucleotide primer has a length of from 20 to 50 bases.

5. The method of claim 3, wherein the first oligonucleotide primer comprises a detectable label or capture moiety.

6. The method of claim 3, further comprising the step of introducing a second D-loop by hybridizing the duplex DNA molecule with a second oligonucleotide primer which is substantially complementary to a second initiation site, said target region lying between the first and second initiation sites.

7. The method of claim 6, wherein the first and second oligonucleotide primers each have a length of from 20 to 50 bases.

8. The method of claim 6, wherein at least one of the oligonucleotide primers comprises a detectable label or capture moiety.

9. The method of claim 6, wherein the replication is performed in a supporting matrix.

10. The method of claim 6, wherein the replisome is assembled via the action of primosomal proteins, single-stranded DNA-binding protein and the DNA polymerase III holoenzyme.

11. The method of claim 10, wherein the primosomal proteins includes a mutant PriA protein which lacks ATPase and helicase functionality.

12. The method of claim 2, wherein the replication is performed in a supporting matrix.

13. The method of claim 1, wherein the replication is performed in a supporting matrix.

14. The method of claim 1, wherein the replisome is assembled via the action of primosomal proteins, single-strand binding protein and holoenzyme III.

15. The method of claim 14, wherein the primosomal proteins includes a mutant PriA protein which lacks ATPase and helicase functionality.

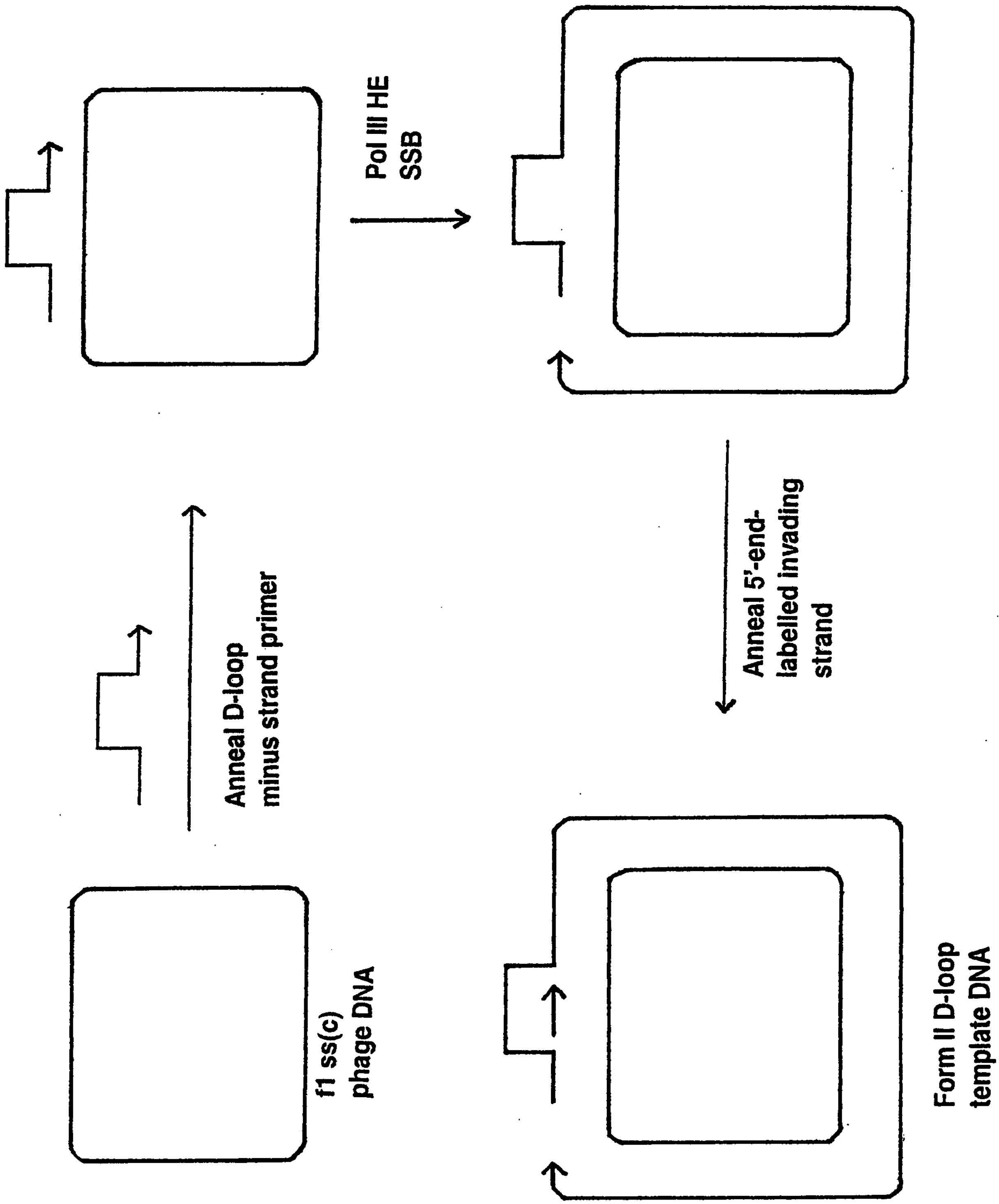
f1 ss(c)
phage DNA
Anneal D-loop
minus strand primer
Pol III HE
SSB
Anneal 5'-end-
labelled invading
strand
Form II D-loop
template DNA

FIG. 1

SEQUENCE LISTING

<110> Marians, Kenneth J.
Joing, Liu

<120> Process for DNA Replication

<130> MSK.P-041-WO

<140>
<141>

<150> 60/118,703
<151> 1999-02-04

<160> 6

<170> PatentIn Ver. 2.1

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<213> Escherichia coli

<220>
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ccgtcacaat caatagaaaa ttcatatggt ttaccagcgc 100

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<211> 42
<212> DNA
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<220>
<223> minus strand oligonucleotide

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<220>
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<210> 5
<211> 24
<212> DNA
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<220>
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<212> DNA
<213> Escherichia coli

<220>
<223> BamHI primer

<400> 6
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PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

To: PARSONS, NANCY
OPPEDAHL & LARSON LLP
P. O. BOX 5270
611 MAIN STREET
FRISCO, CO 80443-5270
UNITED STATES OF AMERICA

FILE COPY 220

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

Form PCT/ISA/220 (July 1998) DO NOT MAIL

	Date of Mailing *(day/month/year)*
Applicant's or agent's file reference MSK.P-041-WO	**FOR FURTHER ACTION** **See paragraphs 1 and 4 below**
International application No. PCT/US00/04445	International filing date *(day/month/year)* 03 FEBRUARY 2000
Applicant SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH	

1. [X] The applicant is hereby notified that the international search report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the international search report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in rules 90 *bis* 1 and 90 *bis* 3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

BRADLEY L. SISSON
PRIMARY EXAMINER
GROUP 1800

Facsimile No.	Authorized officer AND Telephone No.
(703) 305-3230	FRANK LU (703) 308-1235

PATENT COOPERATION TREATY

FILE COPY 210

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Form PCT/ISA/210 (first sheet) (July 1998) **DO NOT MAIL**

Applicant's or agent's file reference MSK.P-041-WO	**FOR FURTHER ACTION**	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
International application No. PCT/US00/04445	International filing date *(day/month/year)* 03 FEBRUARY 2000	(Earliest) Priority Date *(day/month/year)* 04 FEBRUARY 1999
Applicant SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of ____ sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing:

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readable form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (See Box II).

4. With regard to the **title**,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. ______

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

INTERNATIO[redacted] SEARCH REPORT
Form PCT/ISA/21[redacted]tinuation of first sheet(1)) (July 1998)
FILE COPY - DO NOT MAIL

[redacted]national application No.
[redacted]CT/US00/04445

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Form PCT/ISA/210 (continuation of first sheet(2))(July 1998)

FILE COPY - DO NOT MAIL

International application No.

PCT/US00/04445

Box III TEXT OF THE ABSTRACT (Continuation of item 5 of the first sheet)

INTERNATIO SEARCH REPORT
Form PCT/ISA/210 cond sheet) (July 1998)
FILE COPY - DO NOT MAIL

ational application No.
T/US00/04445

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12Q 1/68; C12P 19/34; C12N 9/00
US CL :435/6, 91.1, 91.32, 183
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.1, 91.32, 183, 7.32; 436/94; 536/23.1, 23.7, 23.72, 24.3, 24.33, 25.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN and WEST
D-loop, DNA replication, pri A, replisome, helicase, primosome, primosomal proteins

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CAPS Janniere et al. Replication terminus for DNA polymerase I during initiation of pAMbeta1 replication: role of the plasmid-encoded resolution system. Molecular Microbiology, 1997, Vol. 23, No.3, 525-535, page 525-535, especially pages 525-527 and 533.	1-4 and 14
Y	CAPS MaGlynn et al., The DNA replication protein PriA and the recombination protein RecG bind D-loops. J. Mol. Biol. 1997, Vol. 270, Pages 212-221, especially pages 212-214 and 217-220.	1-4, 6, 7, 10 and 14
Y	CAPS Karet et al., Quantification of mRNA in human tissue using fluorescent nested reverse-transcriptase polymerase chain reaction. Anal. Biochem. 1994, Vol 220, Page 384-390, especially pages 385 and 386.	1-10 and 12-14

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date		
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search 06 APRIL 2000	Date of mailing of the international search report
Facsimile No. (703) 305-3230	Authorized officer AND Telephone No. FRANK LU BRADLEY L. SISSON PRIMARY EXAMINER GROUP 1800

INTERNATION[redacted] SEARCH REPORT
Form PCT/ISA/21[redacted]ntinuation of second sheet) (July 1998)
FILE COPY - DO NOT MAIL

[redacted]rnational application No.
[redacted]T/US00/04445

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Caps Masai et al., Escherichia coli PriA protein is essential for inducible and constitutive stable DNA replication. EMBO J. 1994, Vol.13, No. 22, Page 5338-5345, especially pages 5338, 5339, 5344 and 5345.	1-4, 6, 7, 10 and 14
Y	Caps Al-Deib et al. Modulation of recombination and DNA repair by the recG and PriA helicases of Escherchia cili K-12. J. Bacteriol. December 1996, Vol. 178, No. 23, Page 6782-6789.	1-4, 6, 7, 10, 11, 14 and 15

INTERNATIONAL SEARCH REPORT

Information on patent family members

Form PCT/ISA/210 (patent family annex) (July 1998)

FILE COPY - DO NOT MAIL

International application No.

PCT/US00/04445

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

To:

NR

PARSONS, Nancy
Oppedahl & Larson LLP
P.O. Box 5068
Dillon, CO 80435-5068
ETATS-UNIS D'AMERIQUE

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

Date of mailing (day/month/year) 30 August 2000 (30.08.00)	
Applicant's or agent's file reference MSK.P-041-WO	**IMPORTANT NOTIFICATION**
International application No. PCT/US00/04445	**International filing date** (day/month/year) 03 February 2000 (03.02.00)

1. The following indications appeared on record concerning:

☐ the applicant ☐ the inventor ☒ the agent ☐ the common representative

Name and Address	State of Nationality	State of Residence
PARSONS, Nancy Oppedahl & Larson LLP P.O. Box 5270 611 Main Street Frisco, CO 80443-5270 United States of America		
	Telephone No. 970-668-2050	
	Facsimile No. 970-668-2082	
	Teleprinter No.	

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

☐ the person ☐ the name ☒ the address ☐ the nationality ☐ the residence

Name and Address	State of Nationality	State of Residence
PARSONS, Nancy Oppedahl & Larson LLP P.O. Box 5068 Dillon, CO 80435-5068 United States of America		
	Telephone No. 970-468-6600	
	Facsimile No. 970-668-0104	
	Teleprinter No.	

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

☒ the receiving Office
☒ the International Searching Authority
☐ the International Preliminary Examining Authority
☒ the designated Offices concerned
☐ the elected Offices concerned
☐ other:

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland** Facsimile No.: (41-22) 740.14.35	Authorized officer Dominique DELMAS Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 04 October 2000 (04.10.00)	
International application No. PCT/US00/04445	**Applicant's or agent's file reference** MSK.P-041-WO
International filing date (day/month/year) 03 February 2000 (03.02.00)	**Priority date** (day/month/year) 04 February 1999 (04.02.99)
Applicant MARIANS, Kenneth et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

09 August 2000 (09.08.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was [ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland** Facsimile No.: (41-22) 740.14.35	Authorized officer Juan Cruz Telephone No.: (41-22) 338.83.38

4

REC'D 23 FEB 2001
WIPO PCT

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference MSK.P-041-WO	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US00/04445	International filing date (*day/month/year*) 03 FEBRUARY 2000	Priority date (*day/month/year*) 04 FEBRUARY 1999
International Patent Classification (IPC) or national classification and IPC IPC(7): C12Q 1/68; C12P 19/34; C12N 9/00 and US Cl.: 435/6, 91.1, 91.32, 183		
Applicant SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets.

   ☑ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 1 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of report with regard to novelty, inventive step or industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand 09 AUGUST 2000	Date of completion of this report 17 JANUARY 2001
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Dorothea Lawrence for FRANK LU Telephone No. (703) 308-1235

Form PCT/IPEA/409 (cover sheet) (July 1998) ★

INTERNATIONAL PRELIMINARY EXAMINATION REPORT	International application No. PCT/US00/04445

I. Basis of the report

1. With regard to the **elements** of the international application:*

☐ the international application as originally filed

☒ the description:
pages (See Attached) ____, as originally filed
pages ____, filed with the demand
pages ____
pages ____, filed with the letter of ____

☒ the claims:
pages (See Attached) ____, as originally filed
pages ____, as amended (together with any statement) under Article 19
pages ____, filed with the demand
pages ____
pages ____, filed with the letter of ____

☒ the drawings:
pages (See Attached) ____, as originally filed
pages ____, filed with the demand
pages ____
pages ____, filed with the letter of ____

☒ the sequence listing part of the description:
pages (See Attached) ____, as originally filed
pages ____, filed with the demand
pages ____
pages ____, filed with the letter of ____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language ____ which is:

☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

☐ the language of publication of the international application (under Rule 48.3(b)).

☐ the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

☒ contained in the international application in printed form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ The statement that the information recorded in computer readable form is identical to the writen sequence listing has been furnished.

4. ☒ The amendments have resulted in the cancellation of:

☒ the description, pages NONE

☒ the claims, Nos. NONE

☒ the drawings, sheets/~~fig~~ NONE

5. ☐ This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

* *Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

***Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US00/04445

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. statement

Novelty (N)	Claims	5-15	YES
	Claims	1-4	NO
Inventive Step (IS)	Claims	6-15	YES
	Claims	1-5	NO
Industrial Applicability (IA)	Claims	1-15	YES
	Claims	NONE	NO

2. citations and explanations (Rule 70.7)

Claims 1-4 lack novelty under PCT Article 33(2) as being anticipated by Janniere et al., (Mol. Microbiology 23, 525-535, 1997).

Janniere et al., teach replication terminus for DNA polymerase I during initiation of pAMbeta1 replication. Replication of plasmid pAM beta 1 is initiated by DNA polymerase I (Pol I) and completed by DNA polymerase III holoenzyme contained in the replisome machinery. In this study they reported that initiation of DNA replication generates D-loop structures containing the nascent leading strand paired to its template (page 525, abstract) in a double stranded form and the displaced strand is in the single-stranded form (page 526, right column, third paragraph). The oligonucleotides used to characterize the segments extruded from D-loop replication intermediates have a length of from 20 to 50 bases (page 533, left column, second paragraph). The reaction involving Pol III HE was performed in the presence of ATP and four deoxynucleotides (page 533,right column). This prior art meets the limitations of the claims 1-4.

Response to Arguments

In page 2, third and fourth paragraphs of applicant's Response to Written Opinion, applicant argued that: (1)" Janniere does not disclose a replication system using proteins which are added by man to a developing D-loop. Indeed, Janniere disclose no use for the purified proteins. Furthermore, no real world application of the observation of the replication intermediates is suggested", and (2)"Janniere does not disclose the use of oligonucleotide primer or any other means to introduce a D-loop at a selected location. Indeed, in the Janniere paper, the D loop is generated as a inherent result of the addition of the polymerase, and not as a separate step prior to the assembly of the replisome. There is no targeting of the D loop to a specific initiation site adjacent to a selected target region".

The arguments have been fully considered but they are not persuasive toward the withdrawal of the rejection. First, Janniere et al., (see page 526, right column, third paragraph) (Continued on Supplemental Sheet.)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US00/04445

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Sheet 10

Continuation of: Boxes I - VIII

I. BASIS OF REPORT:

This report has been drawn on the basis of the description,
page(s) 1-15, as originally filed.
page(s) NONE, filed with the demand.
and additional amendments:
NONE

This report has been drawn on the basis of the claims,
page(s) 17, as originally filed.
page(s) NONE, as amended under Article 19.
page(s) NONE, filed with the demand.
and additional amendments:
Claim Page 16, filed with the letter of 13 December 2000.

This report has been drawn on the basis of the drawings,
page(s) 1, as originally filed.
page(s) NONE, filed with the demand.
and additional amendments:
NONE

This report has been drawn on the basis of the sequence listing part of the description:
page(s) 1 and 2, as originally filed.
pages(s) NONE, filed with the demand.
and additional amendments:
NONE

V. 2. REASONED STATEMENTS - CITATIONS AND EXPLANATIONS (Continued):
showed that D loop structure was generated by DNA polymerase I in the initiation of pAMbeta1 replication. it is well known that the replisome is completed by polymerase III and is required for DNA replication(Devlin, Textbook of Biochemistry with clinical correlations, third Edition, see page 671, first paragraph). Therefore, the replisome formation in the presence of assembly proteins is a inherent property of the reference of Janniere et al., and will be considered as a separate step after D loop formation. Second, Janniere et al., clearly showed the use of oligonucleotide primer to introduce a D-loop (see page 533, left column, last paragraph). Third, in response to applicant's argument that the reference failed to show certain features of applicant's invention such as "no real world application of the observation of the replication intermediates is suggested" by Janniere et al., and "there is no targeting of the D loop to a specific initiation site adjacent to a selected target region" is suggested by Janniere et al., it is noted that the features upon which applicant relies above are not recited in the claim(s). Although the claims are interpreted in light of the specification, limitations from the specification are not read into the claims.

Claims 1-5 lack an inventive step under PCT Article 33(3) as being obvious over Janniere et al., (Mol. Microbiology 23, 525-535, 1997)in view of Karet et al., (Anal. Biochem. 220, 384-390, 1994).

The teachings of Janniere et al., have been summarized previously, *supra*. This prior art meets the limitations of claims 1-4.

Janniere et al., do not disclose fluorescence labeled primer.

Karet et al., teach fluorescence labeled primer (page 384, abstract).

It would have been obvious to one having ordinary skill in the art at the time the invention was made to have used performed the method for replication of a target region of a target DNA molecule as suggested by Janniere et al., using a fluorescence labeled primer. The prior art provided by Karet et al., would have motivated one having ordinary skill in the art to perform the method for replication of a target region of a target DNA molecule using a fluorescence-labeled primer. One having ordinary skill in the art at the time the invention was made would have been a reasonable expectation of success to combine these prior arts together because all of prior art are known and are easy to use.

Claims 6-15 meet the criteria set out in PCT Article 33(2)-(4), because the prior art does not teach or fairly suggest the limitations of claims 6-15.

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Sheet 11

Continuation of: Boxes I - VIII

Response to Arguments

In page 2, last paragraphs of applicant's Response to Written Opinion, applicant argued that the teachings of Janniere et al., was "insufficient to cure the deficiencies of the Janniere paper".

The argument has been fully considered but it is not persuasive toward the withdrawal of the objection because, as shown above, the teachings of Janniere et al., clearly meets the limitations of the claims 1-4. There is no deficiency of the Janniere paper as suggested by applicant.

-------------------- NEW CITATIONS --------------------

DEVLIN,Thomas. Textbook of biochemistry with clinical correlation. Wiley-Liss, Inc., 605 Third Avenue, New York, NY10158-0012. 1992, pages 669-671, especially page 671.

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(54) Title: PROCESS FOR DNA REPLICATION

(57) Abstract

A method is provided for replicating DNA, and in particular for replicating large segments of DNA. A primer is combined with a target DNA molecule to be replicated. The primer is designed to be at least partially homologous to a known site on the target DNA, and to create a D-loop when hybridized with that site. A replisome is then assembled at the D-loop, and this replisome creates a copy of the DNA, starting at the primer binding site. By utilizing two species of D-loop primers which bind to remote sites on the DNA flanking a region to be replicated, large sections of DNA can be replicated in a manner comparable to PCR. The replicated DNA can be analyzed to detect variations in the genetic sequence of the target, for linkage mapping and as a source of longer DNA molecules having a desired sequence.

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PROCESS FOR DNA REPLICATION

This application was supported by NIH Grant No. GM34557. The United States may have rights under this application.

This application claims priority from US Provisional Application No. 60/118,703, which application is incorporated herein by reference for those countries where such incorporation is allowed.

Background of the Invention

This application relates to a process for DNA replication, and to the application of this process for a variety of purposes.

Replication of DNA and other nucleic acids is a complex natural phenomenon which occurs within all biological systems. To facilitate the exploitation of the resources represented in the diverse genetic materials of the world's organisms, however, it is desirable to be able to replicate selected DNA sequences under more controlled conditions, for example to produce increased amounts of one sequence. Such replication of selected DNA sequences is required for a great many applications of potential scientific and industrial significance, and has been accomplished by a variety of techniques. These include cloning of the DNA sequences into plasmids or genes, and replication of the plasmid using the DNA replication mechanisms of a host organism, and amplification techniques such as PCR or ligase amplification. Cloning is capable of replicating complete gene sequences, but requires the introduction of the sequences into a host organism, and the subsequent recovery of the duplicated DNA. PCR and similar amplification techniques offer increased flexibility, including the ability to introduce labels and/or sequence variations into the replicated DNA, and avoid the use of a host organism, but are limited in the length of the sequence which can be replicated. Thus, there remains a need for a methodology which will permit the replication of long DNA molecules, while providing the flexibility associated with PCR amplification. It is an object of the present invention to provide such a methodology.

Summary of the Invention

The present invention provides a method for replicating DNA, and in particular for replicating large segments of DNA. In accordance with the invention, a primer is combined with a target DNA molecule to be replicated. The primer is designed to be at least partially homologous to a known site on the target DNA, and to create a D-loop when hybridized with that site. A replisome is then assembled at the D-loop, and this replisome creates a copy of the DNA, starting at the primer binding site. By utilizing two species of D-loop primers which bind to remote sites on the DNA flanking a region to be replicated, large sections of DNA can be replicated in a manner comparable to PCR.

The replicated DNA can be analyzed to detect variations in the genetic sequence of the target, for linkage mapping and as a source of longer DNA molecules having a desired sequence.

Brief Description of the Drawing

Fig. 1 shows the scheme used for making a double-stranded circular template DNA molecule containing a D-loop, which was used to validate the concept of the invention.

Detailed Description of the Invention

The present invention provides a method for the controlled replication, generally *in vitro*, of selected regions of DNA. In accordance with the invention, replication of a target region of a target DNA molecule is accomplished by:

(a) introducing a D-loop into the target DNA molecule at a selected initiation point adjacent to the target region;

(b) assembling a replisome at the D-loop; and

(c) providing DNA monomers (dNTPs) and ATP, whereby the target region is replicated. ATP is preferably provided at concentrations in excess of about 1 mM. ATP is required because the formation of a processive DNA polymerase complex requires ATP hydrolysis and also because DnaB, the DNA helicase, requires concentration in excess of 1 mM to be maximally active.

Introduction of a D-loop at a selected initiation site in duplex DNA can be accomplished using an oligonucleotide primer which hybridizes with double-stranded DNA at a selected initiation site. The non-hybridized strand is displaced to create the D-loop. D-loop formation can be driven by the homologous pairing enzyme, RecA, as has been described in the literature. See, McEntee et al., *Proc. Nat'l Acad. Sci. (USA)* 76: 2615-2619 (1979), which is incorporated herein by reference. D-loop formation could also be driven by other methods, for example heating at a moderately high temperature (for example 75-80°C) may be enough to drive annealing, particularly in regions rich in A+T bases.

The oligonucleotide primer which is used for generation of the D-loop generally has a length of from 20 to about 50 bases. The primer is selected to be substantially complementary to one of the two strands of the target DNA duplex at the initiation site. As used herein, the term "substantially complementary" refers to a primer which will hybridize with the target DNA duplex under conditions of moderately high stringency. However, it will be appreciated that RecA mediated hybridization, if employed, is an enzymatic strand-pairing reaction, and that conditions normally used for DNA-DNA hybridization (e.g. 0.6 M NaCl) would actually be inhibitory. Thus the precise conditions corresponding to "moderately high stringency" may vary depending on the methodology used to drive the annealing. In a general sense, however, the term "substantially complementary" includes (1) primers which are perfectly complementary to the target DNA molecule, (2) primers which are complementary for most of their length, but which include one or several mismatches from perfect complementarity, although not enough mismatches to significantly reduce hybridization specificity; and (3) degenerate primers which include several bases at a given site to accommodate a multiplicity of common alleles in the target DNA. The use of mismatched primers may result from the presence of a mutation in the initiation site, or the mismatch may be intentionally selected for introduction of a desired sequence variation into the replicated DNA.

The primers used in the invention may also include one or more non-hybridized regions for the purpose of introducing a desired additional sequence into the replicated DNA. For example, this additional sequence may be a sequence which introduces a restriction site near the end of the replicated DNA to facilitate insertion of the replicated copies into other DNA molecules. Preferred restriction sites will be those recognized by

rare-cutting restriction enzymes which generally recognize 8-base sequences, or intron-homing endonucleases such as PI-SceI from yeast which recognizes a 31-base pair sequence. This will reduce the likelihood of cleavage occurring within the replicated DNA at other than the intended cleavage site.

In an alternative embodiment of the invention useful with single-stranded templates, the primer used comprises a 3'- and a 5' region which are substantially complementary to portions of the target DNA template, and a central non-complementary region which forms a D-loop when the primer is hybridized with the target DNA. A second primer which is complementary is used to form the invading strand of the D-loop. Similar variations for insertion of cleavage sites etc, may be incorporated in the structure of such primers.

The primers used in the method of the invention may also include a detectable label or capture moiety. Suitable detectable labels and capture moieties are well known in the art as comparable materials are used in PCR, nucleic acid sequencing, and hybridization-based assays. Specific, non-limiting examples of suitable labels and/or capture moieties include fluorescent dyes such as fluorescein, Texas Red or cyanine dyes; enzyme labels such as alkaline phosphatase; and capturable labels such as biotin. Nucleic acid tails which specifically interact with a known capture sequence can also be employed.

In a preferred embodiment of the invention, the primer is combined with target double-stranded DNA under conditions suitable for hybridization and in the presence of the enzyme RecA, which results in the formation of a D-loop at the site of primer binding. Unlike common *in vitro* processes such as PCR, which utilize bacterial polymerases of inherently low processivity, the present invention utilizes replisomes. Replisomes are multi-protein associations which form at a replication fork and act in concert to replicate DNA. Replisomes provide much greater processivity than polymerases used for PCR. For example, the *E. coli* replisome can synthesize pieces of DNA at least as long as a megabase (1×10^6 nucleotides). The fidelity of copying is also quite high, with the *E. coli* replisome making fewer than 1 mistake in 10^8 nucleotides synthesized. Furthermore, unlike PCR, replisomes are substantially insensitive to regions of secondary structure in the DNA template. Thus, utilization of replisomes offers numerous advantages over the use of polymerases.

Replisomes include proteins which perform a variety of functions. Replication of DNA using replisomes depends on an initial unwinding of the DNA duplex at an origin of replication, and the continued unwinding along the strands as the replication process proceeds. This unwinding is carried out by DNA helicases. The resultant regions of single-stranded DNA are stabilized by the binding of single-stranded DNA-binding proteins which are also part of the replisome. The stabilized single-stranded regions are then accessible to the enzymatic activities of polymerases enzymes required for replication to proceed.

Replisomes have been shown to be substantially self assembling. Thus, when the necessary proteins are present under appropriate conditions, the replisome will assemble. We have found that this assembly will occur at a D-loop. A preferred combination of proteins for formation of a replisome in accordance with the present invention includes the following proteins:

PriA, PriB, PriC, DnaT, DnaB, DnaC (primosomal proteins);

single-stranded DNA-binding protein (SSB); and

DNA polymerase III holoenzyme (Pol III HE).

An alternative combination utilizes the mutant protein DnaC810, (described below) in place of PriA, PriB, PriC and DnaT.

The preparation and recovery of these various proteins is well described in the art, including the art cited below which is incorporated herein by reference. Pol III HE may be used in a form recovered directly by purification from *E. coli*, or as a combination of Pol III* and the β subunit. Pol III HE may also be reconstituted from individually overexpressed and purified subunits. These subunits are α (DnaE), ϵ (DnaQ), θ (HolE), β (DnaN), τ (DnaX, full length), γ (DnaX, truncated), δ (HolA), δ' (HolB), χ (HolC) and ψ (HolD). Preparation of Pol III HE is described in US Patents Nos. 5,668,004 and 5,583,026 which are incorporated herein by reference for those countries in which such incorporation is permitted.

Replisomes have been found to initiate DNA replication at the site of a D-loop. Thus, the D-loop formed by the interaction of the primer with the target DNA molecule serves as the initiation site for the replication process in accordance with the invention. When appropriate nucleic acid monomers (i.e., deoxynucleotide triphosphates,

dATP, dCTP, dGTP and dTTP) and ATP are available, a copy of the strand of the DNA molecule to which the primer hybridizes is produced. The length of replicated material which can be produced in this way is much greater than the length which can be produced using PCR or comparable techniques, with lengths in excess of 5000-500,000 bases being readily attainable. Thus, the method provides the ability to make copies of entire large genes, including both intron and exon sequences.

As will be apparent to persons skilled in the art, a person making copies of DNA will generally be interested in obtaining those copies of a particular region of the DNA, which is referred to herein as the "target region." The target region may be a particular gene, or a particular portion of a gene depending on the use for which the copied DNA is intended. The ability to produce copies of very large numbers of bases changes the practical limits on the proximity between the primer and the target region from those which are usually observed in the PCR and comparable methods. Thus, while the initiation site must be "adjacent" to the target region, this means only that the initiation site must be close enough to and on the correct side of the target region such that a replisome assembled at the D-loop will copy the DNA of the target region.

In a preferred embodiment of the invention, two primers are utilized. The first primer is as described above, and hybridizes with a first strand of a double stranded DNA duplex. The second primer also is a substantially complementary oligonucleotide primer, but it hybridizes to the second strand of the DNA duplex at a second initiation site located on the other side of the target region. Thus, the two primers flank the target region, in the same manner that PCR primers flank a region to be amplified. Further, the same principle which leads to amplification of just the region bounded by PCR primers, leads to creation of much larger pieces of replicated DNA spanning the region between the two initiation sites using the method of the invention, although the efficiency may not be as great as achieved with PCR. This reduced efficiency is less of a problem than one might expect, however, since the large size of the replicated DNA makes them inherently more detectable than small fragments. On the other hand, since the process of the invention works on double-stranded DNA, it is not necessary to separate the strands of the target and the newly replicated DNA before proceeding with the next cycle.

While the large size of the replicated DNA offers advantages for purposes of detection, it may also pose problems. Very large DNA molecules (i.e., those that are hundred of kilobases in length) are fragile, and may be broken if manipulated in simple solutions. Thus, production of fragments of such lengths, and meaningful analysis of the lengths of such fragments may require that the reaction be performed in a supporting matrix, such as an agarose gel. Replicated DNA can be transferred out of the supporting matrix, for example for introduction into a matrix for separation based on size by electrophoresis.

DNA replicated in accordance with the invention may be utilized for a variety of purposes. First, the replicated DNA may be used as a source of genetic material to be spliced into still larger nucleic acid constructs, including plasmids, cosmids, viral vectors etc., to facilitate expression of the replicated DNA in a suitable host system. Such splicing can be facilitated by the incorporation of restriction sites near then ends of the replicated DNA as discussed above. When two primers are utilized, restriction sites can be introduced at both ends of the replicated DNA.

Second, the replication of DNA in accordance with this method can be used as part of a method for detecting genomic rearrangements in a target DNA sequence. In such a method, a D-loop is introduced into the DNA at a selected initiation point, a replisome is assembled at the D-loop, and the DNA is copied to produce sufficient numbers of copies for analysis. The copied product is analyzed to detect variations in size or organization of the copied material using size-specific separations, hybridization probes and other standard analytical techniques. It will be appreciated that the use of size-specific separations requires the production of a product of defined lengths, and thus will generally require the use of the two primer embodiment discussed above. On the other hand, where the analysis involves the measurement of the interaction of the DNA with a labeled or immobilized probe, the replication of multiple copies of a single strand of the DNA, without amplification, may be sufficient.

Third, the method can be used to facilitate linkage mapping. For example, the method can be used in the circumstance where two chromosomal markers are known to be near one another, but where the exact distance separating them is not known. D-loop oligonucleotide primers are synthesized for each marker for both the DNA strands. Combinations of the primers are used to replicate the region between the two markers, and

the size of the product formed reflects the chromosomal distance between the two markers. The method may also be used to map unlinked genes, and markers such as RFLPs, SNIPs and ESTs.

To demonstrate the ability of the replisomes to assemble at a D loop and replicate the DNA, we used a small bacteriophage DNA molecule as a model system as described in the following non-limiting examples. The conditions for replisome assembly and DNA replication can be extended to use with larger molecules, and with substantially complementary primers as discussed above.

EXAMPLE 1

Preparation of DNA Replication Proteins

To prepare DnaC810, a *dnaC810* open reading frame was constructed by splicing overlap extension polymerase chain reaction and cloned into the *Nde*I site of the pET11C overexpression plasmid (Novagen). Overexpression and purification of DnaC810 was as for the wild type protein.

PriA, PriB, PriC, DnaT, DnaB and DnaC were purified by the methods described in Marians, K.J. *Methods Enzymol.* 262: 507-521 (1995). SSB was purified using the procedures described in Minden and Marians, *J. Biol. Chem.* 260: 9316-9325 (1985). The DNA polymerase III holoenzyme was either reconstituted from Pol III* and β subunit as described by Wu et al. *J. Biol. Chem.* 267: 4030-4044 (1992) or from purified subunits as described in Marians et al., *J. Biol. Chem.* 273: 2452-2457 (1998).

EXAMPLE 2

To validate the operability of the inventive concept, a double-stranded circular template DNA was prepared in accordance with the steps shown in Fig. 1. A 100 nt-long oligonucleotide primer (Seq. ID No. 1) was annealed to f1R408 viral DNA (Russell et al., *Gene* 45: 333-339 (1986)). The central 42 nt of this oligonucleotide are non-homologous with the template, thus forming a D-loop in the resulting heteroduplex. Incubation of the heteroduplex with DNA Polymerase III holoenzyme in the presence of SSB and DNA monomers resulted in the extension of the primer and the formation of a nicked form II DNA with a 42 nt-long bubble region. During the last two minutes of this

incubation, ddTTP and ddATP were introduced at concentrations 20-fold higher than dTTP and dATP to ensure that complementary strand synthesis could not be extended further. After phenol extraction and ethanol precipitation, the DNA products were purified by electrophoresis through native agarose gels. Complete form II bubble DNA was recovered from the gel and a [5'-^{32}P] minus strand oligonucleotide (Seq. ID. No. 2) was then annealed to the D loop form II template. The template was then gel filtered through Biogel A5M to remove unannealed oligonucleotide and unincorporated [γ-^{32}P] ATP.

EXAMPLE 3

Reaction mixtures (12 μl) containing 50 mM Hepes-KOH (pH 8.0), 10 mM MgOAc, 10 mM DTT, 80 mM KCl, 200 μg/ml bovine serum albumin, 2 mM ATP, 40 μM dNTPs, 0.42 nM [^{32}P] form II D loop DNA template, 0.5 μM SSB, 225 nM DnaC, 30 nM DNA polymerase III holoenzyme, PriA, PriB, PriC, DnaT and DnaB were incubated at 37°C for 10 minutes. To test the sufficiency of various combinations of proteins to replicate the template prepared in Example 2, reactions were also performed in which one of the proteins (PriA, PriB, PriC, DnaT, DnaC and DnaB) was omitted in each reaction mixture. As controls, template alone and template with the holoenzyme alone were also evaluated. Reactions were terminated by the addition of EDTA to a concentration of 25 mM and NaOH to a concentration of 50 mM. The reaction products were evaluated by electrophoresis at 2 V/cm for 20 hours at room temperature through horizontal 0.7% alkaline agarose gels using 30 mM NaOH, 2 mM EDTA as the electrophoresis buffer. The gels were neutralized, dried and analyzed by autoradiography.

The electrophoresis gels showed that incubation of the D-loop template, the seven primosomal proteins, SSB and DNA polymerase III holoenzyme resulted in extension of the invading strand oligonucleotide (42 nt, Seq. ID. No. 2) to the full length template size (6.4 kb). The efficiency of the reaction varied, but generally 15-30% of the invading strand could be elongated to full length in a 10 minute incubation. The reaction exhibited an absolute requirement for all of the primosomal proteins except PriC. Omission of this protein resulted in a decrease in DNA synthesis to one-third that of the complete reaction. This observation was similar to those reported for replication on different templates. Ng et al., *J. Biol. Chem.* 271: 15642-15648 (1996). Some extension of the invading strand by the

holoenzyme alone could be observed, but this was suppressed by the presence of PriA. If the invading strand was omitted from the reaction, and [α-^{32}P] dATP was included, no DNA replication could be observed.

EXAMPLE 4

Because DNA helicases were being introduced to the DNA during primosome assembly, extension of the invading strand could result from one of two processes: either (1) assembly of a *bona fide* replication fork at the D loop followed by elongation of the leading strand coupled with unwinding of the duplex DNA template, or (2) uncoupled unwinding of the template DNA leaving an oligonucleotide annealed to the viral single stranded DNA that could be elongated in a primer extension reaction by the polymerase. We previously showed that coupled replication fork action requires a protein-protein interaction between DnaB and the τ subunit of the holoenzyme. Kim et al.., *Cell* 84: 643-650 (1996). In the presence of this interaction, replication forks could move rapidly, at nearly 1000 nt/sec, whereas in its absence, the polymerase becomes stuck behind a slow-moving helicase and replication fork progression proceeds at only about 30 nt/sec.

To evaluate the mechanism active in the replication of DNA in the method of the invention, the speed of elongation of the invading strand was assessed in the presence and absence of τ using holoenzyme reconstituted from individual purified subunits. Ten second time points were taken from the start of the reaction, and the elongated products were examined on denaturing gels. Full length material could be observed in the presence of τ after 10 seconds, whereas even after 60 seconds no full length material was observed in its absence. This corresponds to a rate of replication fork progression in the presence of τ of 600-700 nt/sec, similar to what has been observed in the past for other replication systems. Mok et al., *J. Biol. Chem.* 262: 16644-16654 (1987). Thus, we conclude that *bona fide* replication fork assembly occurs at the D loop on the template in the presence of primosomal proteins, SSB and the holoenzyme.

EXAMPLE 5

All of the phenotypes of *priA* null mutations can be suppressed by mutated *priA* alleles that encode PriA proteins that are no longer ATPases or DNA helicases, but still

catalyze primosome assembly. Zavitz et al., *J. Biol. Chem.* 267: 6933-6940 (1992). These mutations are substitutions in the invariant Lys in the Walker A box nucleotide-binding motif. If the PriA-dependent replication fork assembly described here were relevant to what happened in the cell, we would expect these mutant proteins to substitute fully for wild-type PriA in the replication reaction. To test this, three mutant proteins, having the K230R, K230A and K230D substitutions were tested. All three supported replication on the D loop to a greater extent than the wild-type protein. This same type of improved activity in the mutant proteins has been observed in other systems (Zavitz, *supra*), and may arise because the mutant proteins remain bound to the site of DNA binding, providing a better target than the wild-type protein that can move off the site because of its helicase activity.

EXAMPLE 6

E. coli strains carrying *priA* mutations are very difficult to grow. They are rich-media sensitive, form huge filaments, and have a viability roughly one-hundredth that of the wild-type. Sandler et al., *Genetics* 143: 5-13 (1996); Nurse et al., *J. Bacteriol.* 6686-6693 (1991); Masai et al., *EMBO J.* 13: 5338-5345 (1994). Suppressor mutations that restore viability, as well as ablate constitutive induction of the SOS response and the defects in homologous repair of UV-damaged DNA, arise overnight after transduction of the *priA2:kan* allele into fresh recipient cells. The mutations map to *dnaC*. (Sandler, *supra*). DnaC forms a complex with DnaB in solution (Wicker et al., *Proc. Natl. Acad Sci. (USA)* 72: 921-925 (1975), and is required for the efficient transfer of DnaB to DNA in the presence of other replication protein. Marians et al., *Ann. Rev. Biochem.* 61: 673-719 (1992). In order to assess the biochemical properties of these altered DnaC proteins, one such suppressor allele, *dnaC810*, was molecularly cloned into an expression plasmid and the mutant protein purified as described in Example 7, *infra*.

Strains carrying *dnaC810* no longer require PriA for viability. This suggests that if the essential role for PriA in cellular metabolism was to catalyze assembly of replication forks at recombination intermediates, DnaC810 must be able to bypass the requirement for PriA to recognize the D loop and nucleate the assembly of a primosome. Accordingly, we tested whether DnaC810 alone could direct transfer of DnaB to the D loop template DNA.

In the presence of SSB and the holoenzyme, the combination of wild-type DnaC and DnaB did not support elongation of the invading strand of the D loop. On the other hand, DnaC810 was clearly able to load DnaB to the D loop on the template in the absence of the other primosomal proteins, as evidenced by the elongation of the invading strand to full length. Thus, the E176G substitution in DnaC810 represents a true gain of function mutation that allows bypass of the DnaB loading pathway that involves PriA, PriB, PriC and DnaT and permits a reduction in the number of proteins necessary for the practice of the present invention.

Interestingly, the relative efficiencies of the replication reaction catalyzed in the presence of DnaC810 and DnaB varied compared to the reaction catalyzed by the complete set of primosomal proteins. At 80 mM KCl, the DnaC810 reaction was 5- to 10-fold more efficient. However, at 600 mM potassium glutamate, the reaction catalyzed by the complete set of proteins was more efficient by a factor of 2. While not intending to be bound by a particular mechanism, this difference may arise from differences in the relative stability of intermediate complexes that are formed during the loading of DnaB to DNA.

EXAMPLE 7

Construction of Plasmid pET11c-dnaC810—A *dnaC810* open reading frame (ORF) was made by two-step overlapping polymerase chain reaction (PCR) Morton et al., *Gene* 77: 61-68 (1989). The N-terminal coding region of *dnaC810* was PCR amplified using plasmid pET11c-*dnaC* (Marians, K.J, *Methods Enzymol.* 262:m 507-521 (1995)) as a template and two flanking primers:

(i) the NdeI primer (Seq. ID No. 3), which carries a *Nde*I site at the *dnaC* initiator codon, and

(ii) the AgeI′ primer (Seq. ID. No. 4), which carries the designed point mutation (E176G, GAA→GGT). The C-terminal coding region of *dnaC810* was also PCR amplified using plasmid pET11c-*dnaC* as a template and two different flanking primers:

(i) the AgeI primer (Seq. ID No. 5), which is complementary to the AgeI′ primer and

(ii) the BamHI primer (Seq. ID No. 6), which carries a *Bam*HI site just downstream of the *dnaC* stop codon. These overlapping N- and C-terminal fragments were gel purified after PCR and further PCR extended and amplified with the two flanking NdeI and BamHI

primers. The gel purified *dnaC810* ORF fragment was digested with *Nde*I and *Bam*HI and ligated with *Nde*I- and *Bam*HI-digested pET11c plasmid DNA to give pET11c-*dnaC810*.

Purification of DnaC810—Because of the extreme overproduction, DnaC810 was followed during purification by SDS-PAGE. BL21(DE3)pLysS carrying pET11c-*dnaC810* was grown in 12 l L Broth (Mainatis et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1982)) containing 0.4% glucose and 300 mg/ml ampicillin to OD_{600} = 0.4 and then induced in the presence of 1 mM IPTG for 3 h. Cells were chilled, pelleted by centrifugation, and resuspended in 50 mM Tris-HCl (pH 8.4 at 4 °C) and 10% sucrose. The cell suspension (50 ml) was adjusted to 150 mM KCl, 20 mM EDTA, 5 mM dithiothreitol, 0.02% lysozyme, and 0.1% Brij 58 and incubated at 0 °C for 10 min. This suspension was centrifuged at 100,000 × g for 1 h (Sorvall T865 rotor). The supernatant (fraction 1, 65 ml, 3510 mg protein) was adjusted to 0.04% polymin P by dropwise addition of a 1% solution. The precipitate was removed by centrifugation at 47,000 × g in a Sorvall SS-34 rotor for 30 min. The supernatant was further subjected to $(NH_4)_2SO_4$ fractionation (50% saturation) by the addition of solid. The resulting protein pellet was collected by centrifugation at 47,000 × g in a Sorvall SS-34 rotor for 30 min. The protein pellet was resuspended in 8 ml of buffer A [50 mM Tris-HCl (pH 7.5 at 4 °C), 1 mM EDTA, 5 mM dithiothreitol, 20% glycerol, 0.01% Brij 58] + 50 mM NaCl to give fraction 2 (13 ml, 1108 mg protein). Fraction 2 was dialyzed against 2 l of buffer A + 50 mM NaCl for 12 h and then loaded onto a 100-ml DEAE-cellulose column (4 cm x 20 cm) that had been equilibrated previously with buffer A + 50 mM NaCl. The column was washed with 200 ml of buffer A + 50 mM NaCl. Fractions (15 ml) of the flow-through and wash that contained protein were pooled to give fraction 3 (81 ml, 363 mg protein). Fraction 3 was loaded directly onto a 35-ml SP-Sepharose FF column (formed in a 60-ml disposable syringe) that had been equilibrated previously with buffer A + 50 mM NaCl. The column was washed with 200 ml of buffer A + 50 mM NaCl and protein was then eluted with a 350-ml linear gradient of 50-300 mM NaCl in buffer A. DnaC810 eluted at 175 mM NaCl (fraction 4, 24 ml, 25 mg protein). Fraction 4 was then loaded directly onto a 6-ml hydroxylapatite column (packed in a 10-ml disposable syringe) that had been equilibrated previously with buffer A + 200 mM NaCl. The column was washed with 12 ml of equilibration buffer and protein was eluted with a 60-ml linear gradient of 0-400

mM $(NH_4)_2SO_4$ in buffer A + 200 mM NaCl. DnaC810 eluted at 150 mM $(NH_4)_2SO_4$ to give fraction 5 (5.2 ml, 16.5 mg protein). Fraction 5 was concentrated bydialyzing against buffer A + 50 mM NaCl + 30% polyethylene glycol 20,000 and loaded onto a 125-ml Superdex-200 FPLC column that had been equilibrated with buffer A + 50 mM NaCl. The column was eluted at 1 ml/min. Fractions (1 ml) containing DnaC810 were pooled to give fraction 6 (7.5 ml, 9.2 mg protein). Fraction 6 was then loaded onto a 3-ml phosphocellulose column that had been equilibrated with buffer A + 50 mM NaCl. The column was washed with 6 ml of equilibration buffer and protein was eluted with a 60-ml linear gradient of 50-400 mM NaCl in buffer A. DnaC810 eluted at 250 mM NaCl (Fraction 7, 3.5 ml, 5.2 mg protein).

Sequence Listing

(Seq. ID No. 1)	ACATACATAA AGGTGGCAAC GCCATTCGAA ATGAGCTCCA TATGCTAGCT AGGGAGGCCC CCGTCACAAT CAATAGAAAA TTCATATGGT TTACCAGCGC
(Seq. ID No. 2)	ATATAAAAGA AACGCAAAGA CACCACGGAA TAAGTTTATT TT
(Seq. ID No. 3)	TAATGCAGGC CATATGAAAA ACGTTGGCGA CCTG
(Seq. ID No. 4)	TCGTATTTCG AACCGGTCTG CACG
(Seq. ID No. 5)	CGTGCAGACC GGTTCGAAAT ACGA
(Seq. ID No. 6)	TTAAGCACTG GGATCCTTAA TACTCTTTAC CTGTTAC

CLAIMS

1. A method for replication of a target region of a target DNA molecule comprising the steps of:

(a) introducing a D-loop into the target DNA molecule at a first initiation point adjacent to the target region;

(b) assembling a replisome at the D-loop; and

(c) providing DNA monomers and ATP to the replisome, whereby the target region is reproduced.

2. The method of claim 1, wherein the target DNA molecule is a duplex DNA.

3. The method of claim 2, wherein the step of introducing a D-loop is performed by hybridizing the duplex DNA molecule with a first oligonucleotide primer which is substantially complementary to the first initiation site.

4. The method of claim 3, wherein the first oligonucleotide primer has a length of from 20 to 50 bases.

5. The method of claim 3, wherein the first oligonucleotide primer comprises a detectable label or capture moiety.

6. The method of claim 3, further comprising the step of introducing a second D-loop by hybridizing the duplex DNA molecule with a second oligonucleotide primer which is substantially complementary to a second initiation site, said target region lying between the first and second initiation sites.

7. The method of claim 6, wherein the first and second oligonucleotide primers each have a length of from 20 to 50 bases.

8. The method of claim 6, wherein at least one of the oligonucleotide primers comprises a detectable label or capture moiety.

9. The method of claim 6, wherein the replication is performed in a supporting matrix.

10. The method of claim 6, wherein the replisome is assembled via the action of primosomal proteins, single-stranded DNA-binding protein and the DNA polymerase III holoenzyme.

11. The method of claim 10, wherein the primosomal proteins includes a mutant PriA protein which lacks ATPase and helicase functionality.

12. The method of claim 2, wherein the replication is performed in a supporting matrix.

13. The method of claim 1, wherein the replication is performed in a supporting matrix.

14. The method of claim 1, wherein the replisome is assembled via the action of primosomal proteins, single-strand binding protein and holoenzyme III.

15. The method of claim 14, wherein the primosomal proteins includes a mutant PriA protein which lacks ATPase and helicase functionality.

8. The method of claim 6, wherein at least one of the oligonucleotide primers comprises a detectable label or capture moiety.

9. The method of claim 6, wherein the replication is performed in a supporting matrix.

10. The method of claim 6, wherein the replisome is assembled via the action of primosomal proteins, single-stranded DNA-binding protein and the DNA polymerase III holoenzyme.

11. The method of claim 10, wherein the primosomal proteins includes a mutant PriA protein which lacks ATPase and helicase functionality.

12. The method of claim 2, wherein the replication is performed in a supporting matrix.

13. The method of claim 1, wherein the replication is performed in a supporting matrix.

14. The method of claim 1, wherein the replisome is assembled via the action of primosomal proteins, single-strand binding protein and holoenzyme III.

15. The method of claim 14, wherein the primosomal proteins includes a mutant PriA protein which lacks ATPase and helicase functionality.

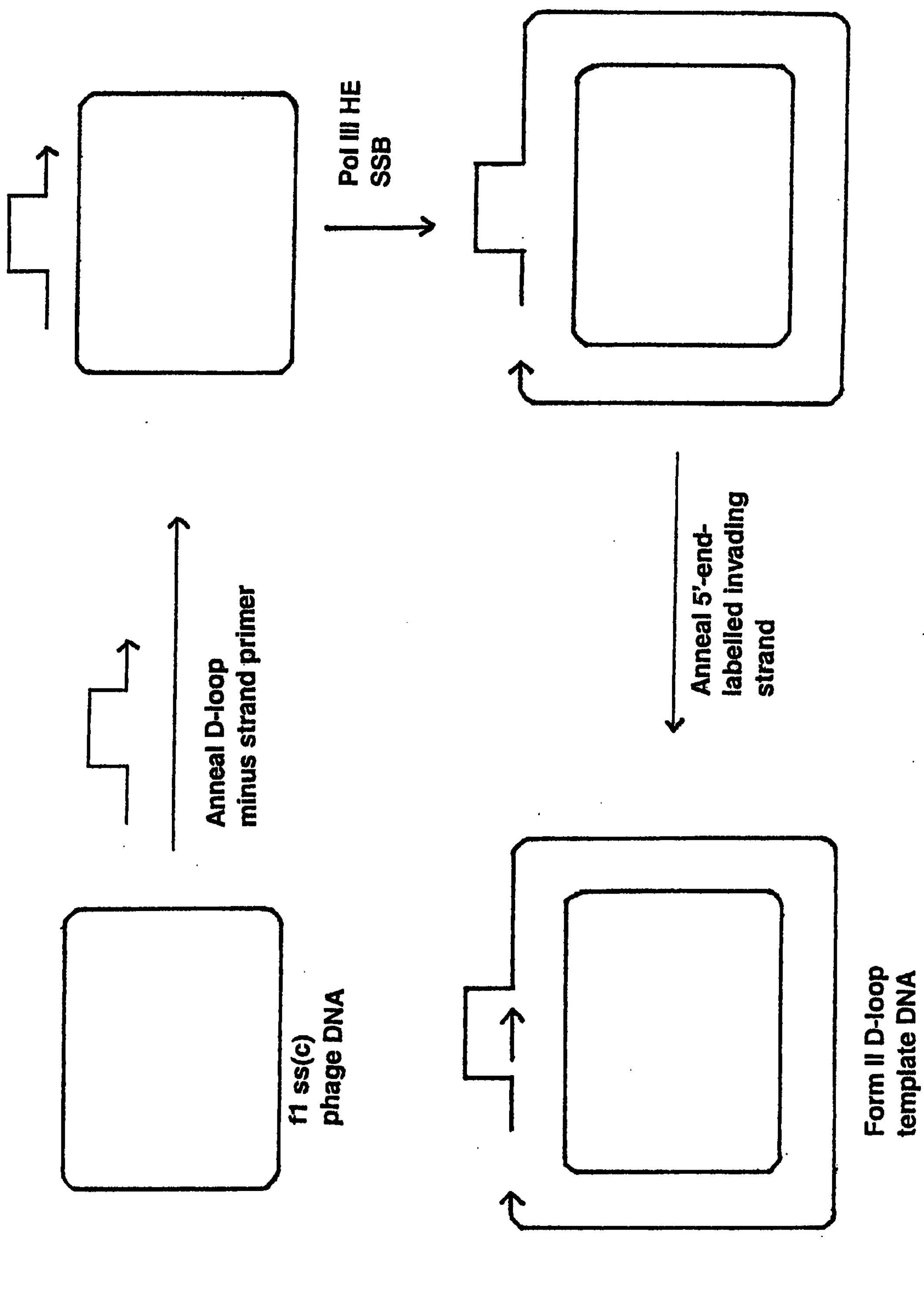
f1 ss(c)
phage DNA
Anneal D-loop
minus strand primer
Pol III HE
SSB
Anneal 5'-end-
labelled invading
strand
Form II D-loop
template DNA

FIG. 1

<220>
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<400> 3
taatgcaggc catatgaaaa acgttggcga cctg 34

<210> 4
<211> 24
<212> DNA
<213> Escherichia coli

<220>
<223> AgeI' primer

<400> 4
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<210> 5
<211> 24
<212> DNA
<213> Escherichia coli

<220>
<223> AgeI primer

<400> 5
cgtgcagacc ggttcgaaat acga 24

<210> 6
<211> 37
<212> DNA
<213> Escherichia coli

<220>
<223> BamHI primer

<400> 6
ttaagcactg ggatccttaa tactctttac ctgttac 37

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/04445

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12Q 1/68; C12P 19/34; C12N 9/00
US CL :435/6, 91.1, 91.32, 183
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.1, 91.32, 183, 7.32; 436/94; 536/23.1, 23.7, 23.72, 24.3, 24.33, 25.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN and WEST
D-loop, DNA replication, pri A, replisome, helicase, primosome, primosomal proteins

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JANNIERE et al. Replication terminus for DNA polymerase I during initiation of pAMbeta1 replication: role of the plasmid-encoded resolution system. Molecular Microbiology. 1997, Vol. 23, No.3, 525-535, page 525-535, especially pages 525-527 and 533.	1-4 and 14
Y	MCGLYNN et al. The DNA replication protein PriA and the recombination protein RecG bind D-loops. J. Mol. Biol. 1997, Vol. 270, Pages 212-221, especially pages 212-214 and 217-220.	1-4, 6, 7, 10 and 14

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance
"B" earlier document published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search
06 APRIL 2000

Date of mailing of the international search report
26 APR 2000

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